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42. (Newly Added) The method of Claim 41 wherein said hepatocyte precursor cell, after having been removed previously from a histocompatible donor, is further genetically engineered *ex vivo* to be capable of treating said liver dysfunction in said human subject.

43. (Newly Added) The method of Claim 21 further comprising administering to the subject progeny of said genetically engineered autologous hepatocyte precursor cell.

REMARKS

Claims 21-23, 25, 27, 29-36, and 39-40 were pending in the application, with Claims 21, 22, and 40 being independent. Claim 22 is hereby cancelled without prejudice to pursuing the subject matter of the cancelled claim in this or other continuing applications. Claims 21, 23, 25, 27, 29, 39, and 40 have been amended. New Claims 41-43 have been added. Upon entry of these amendments, Claims 21, 23, 25, 27, 29-36, and 39-43 will be pending and under active consideration, Claims 21, 40, and 41 being independent. A marked-up version of the claims indicating the changes to the claims is attached hereto as Exhibit A. A copy of all pending claims, as amended, is attached hereto as Exhibit B. The amendments are supported fully by the claims and/or specification as originally filed and, thus, do not represent new subject matter.

Claims 21, 25, 27, 29, and 40 are amended to recite the clarifying term "cell" as recommended kindly by the Examiner.

Claims 23, 25, 27, 29, and 39 are renumbered to correct their dependency.

Claim 25 is amended to recite "progeny thereof, or both" following "precursor cells" in order to comport with the antecedent basis found in Claim 43, from which it depends.

Claim 21 is amended to recite "wherein said hepatocyte precursor cell is removed from

said subject, is genetically engineered *ex vivo* to be capable of treating said liver dysfunction, and is administered” in response to Examiner’s request that the claim be made more clear and complete. Support for this amendment may be found on page 9, lines 12-14, of the specification as filed which recite that “hepatocytes to be genetically modified *ex vivo* can be obtained from a human or non-human animal, modified, and returned to the same human or non-human animal by transplantation or grafting.”

Claim 29 is amended to recite “as a result of the genetic engineering” in response to Examiner’s request for improved clarity.

Claim 40 is amended to remove vagueness and improve clarity, as requested by Examiner.

New Claims 41 and 42 find support throughout the specification and claims as filed, particularly on pages 8-12, and more particularly on page 8, lines 8-15, and page 12, lines 5-7. Further, Applicant notes respectfully that in the Final Office Action, page 12, end of the first full paragraph, the Examiner recites that “the specification teaches use of the precursor cell by itself for treatment of liver dysfunction (in part by supplying to a subject hepatocytes which provide an endogenous gene product which a subject needs).” Support for use of the term “histocompatible” is found inherently in the specification and art as acknowledged by Examiner on page 7 of the Final Office Action, which states that transplantation of tissues from one individual to another who demonstrates a tolerance due to histocompatibility is generally accepted in the art.

New Claim 43 finds support in Claim 21 as filed.

Applicants note the withdrawal of Claims 24, 26, 28, 37 and 38, as requested in Applicants’ Amendment and Response to Final Office Action filed May 5, 2001. Further,

Applicants seek respectfully to clarify the record with regard to the assertion in the both of the Final Office Actions, papers numbered 14 and 17, that Applicants' Amendment filed September 21, 2000, is objected to due to the introduction of the allegedly "new matter" recitation of the term "autologous." In fact, that term was introduced in Applicants' Amendment of May 5, 2001, which, given the withdrawal of the afore-mentioned claims and the new matter objection noted above, will be assumed by Applicants to have been received and considered, despite the absence in the prior record of an explicit statement to such effect.

Inasmuch as the Examiner's Advisory Action pursuant to Applicants' Amendment of May 5, 2001, contained notice that the Information Disclosure Statement (IDS) disclosed in that Amendment failed to comply with 37 C.F.R. § 1.97(d) and that the subject matter contained within was not considered, Applicants include herein, as Exhibit E, a corrected Supplemental IDS including the references, and, for Examiner's convenience, Applicants reiterate below the salient arguments presented in that Amendment with respect to those references for reconsideration in light of the text of the references.

Applicants acknowledge, and thank Examiner for, Examiner's recognition that the present invention is free of the cited art, as noted in the Conclusion of the present Final Office Action, Paper No. 17.

Applicants respectfully request entry of the amendments and remarks made herein into the file history of the present invention. Reconsideration and withdrawal of the rejections set forth in the above-identified Final Office Action are respectfully requested.

I. Rejections Under 35 U.S.C. § 112, First Paragraph

At pages 3-10 of the Final Office Action, paper number 17, Claims 21-23, 25, 27,

29-36, and 39-40 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. The Final Office Action alleges that Claims 21-23, 25, 27, 29-36, and 39-40 encompass a method of treatment of liver dysfunction in a subject comprising administering a genetically engineered hepatocyte precursor to the subject, including a method of *ex vivo* and *in vivo* gene therapy for the treatment of any form of liver dysfunction with a genetically modified hepatocyte precursor cell. The Final Office Actions allege, further, that the claims recite that the administration of said cells may be through injecting, transplanting, or grafting said cells into the subject, in particular the spleen (Claim 25), that a gene of interest can be inserted into the genome of the cell or maintained extrachromasomally (Claims 31 and 32), and recite a list of diseases for which said methodology could be used (Claim 34). In explanation of the rejections, the Final Office Actions detail exhaustively the alleged unpredictability of the arts of gene therapy and, with particular vigor, heterologous and xenotransplantation. Finally, the Final Office Actions allege that the inclusion by Applicants of the term “autologous” constitutes “new matter.”

As alleged in the Final Office Action at the end of the first paragraph on page 5, there are two points of enablement at issue (beside the new matter rejection); first, the amount of guidance and skill in the art to affect treatment of any liver dysfunction by genetically engineering a hepatocyte precursor cell *in vivo* or *ex vivo*, and second, the ability to use the hepatocyte precursor cells in heterologous and xenotransplantation protocols. Applicants traverse respectfully.

A. The New Matter Rejection Should Be Withdrawn

The Final Office Action asserts, at the bottom of page 10, that Claim 21 recites the term “autologous,” but that “these particular embodiments do not have literal nor figurative support in the specification.” Hence, the Final Office Action rejects Claim 21 under 35 U.S.C. § 112, first paragraph. Applicants traverse respectfully.

As noted by Applicant in Applicants’ Amendment of May 5, 2001, literal support for the term “autologous” may indeed be found in the specification as filed. Specification page 10, line 10, in discussing therapeutic uses of hepatocyte precursors of the present invention, recites that such hepatocyte precursors may be used, “in the case of *autologous* grafting, with little risk of immune response and graft rejection” (emphasis added). Support for the term may be found figuratively in the specification on page 9, lines 12-14, which recite that “hepatocytes to be genetically modified *ex vivo* can be obtained from a human or non-human animal, modified, and returned to the same human or non-human animal by transplantation or grafting.” Applicants submit respectfully that one skilled in the art would readily appreciate that this procedure is definitive of autologous transplantation or grafting. Accordingly, Applicants request respectfully that the new matter rejection of Claim 21 under 35 U.S.C. § 112, first paragraph, be withdrawn.

B. Rejections Of The Use The Hepatocyte Precursor Cells In Heterologous And Xenotransplantation Protocols Should Be Withdrawn

Initially, Applicants submit respectfully that Claim 22 has been cancelled herein and, therefore, Applicants submit respectfully that the rejection of Claim 22 under 35 U.S.C. § 112, first paragraph, is rendered moot. With regard to the remaining claims, respectfully, Applicants draw Examiner’s attention to the claims, as amended, which no longer

read on heterologous transplantation and/or xenotransplantation. The claims, as amended, now recite only the use of autologous hepatocyte precursors and histocompatible hepatocyte precursors. Applicants submit respectfully that Examiner's thorough discussion, on pages 7-9 of the Final Office Action, of hyper-acute rejection (HAR) and the alleged ongoing practical difficulties found in the art of heterologous and xeno-transplantation is, accordingly, rendered moot. Applicants note with appreciation Examiner's acknowledgement, on page 7 of the Final Office Action, that transplantation of tissues from one individual to another who demonstrates a tolerance due to histocompatibility is generally accepted in the art; Applicants have amended the claims to comport with this understanding. Accordingly, Applicants request respectfully that rejection of Claims 21, 23, 25, 27, 29-36, and 39-40, based upon alleged deficiencies in the ability to use the hepatocyte precursor cells in heterologous and xenotransplantation protocols, be withdrawn.

C. Rejections Based On Genetically Engineering A Hepatocyte Precursor Cell *In Vivo* And *Ex Vivo* Should Be Withdrawn

As noted above, the Final Office Action alleges, on pages 4-7, that Claims 21-23, 25, 27, 29-36, 39 and 40 encompass a method of treatment of liver dysfunction in a subject comprising administering a genetically engineered hepatocyte precursor to the subject, including a method of *ex vivo* and *in vivo* gene therapy for the treatment of liver dysfunction with a genetically modified hepatocyte precursor cell (Examiner's emphasis), but that the specification is allegedly not enabling for these uses. The Final Office Action further alleges that the specification as filed does not provide any substantive guidance for demonstrating that isolated hepatocyte precursor cells will maintain a precursor like state or will differentiate into

mature hepatocytes *in vitro* or *in vivo*, and that the specification presents only a description for the potential use of the hepatocyte precursor cells in obtaining a genetically engineered hepatocyte precursor and does not demonstrate that one can culture and genetically manipulate said cells either *in vitro* or *in vivo*. Applicants traverse respectfully.

Respectfully, Applicants draw Examiner's attention to the claims, which have been amended so as to delete all references to genetic engineering of hepatocyte precursor cells *in vivo*. As amended, the present claims only encompass genetic engineering of such cells *ex vivo* (*i.e., in vitro*). Accordingly, Applicants submit respectfully that the rejection based on the alleged lack of enablement of genetic engineering cells *in vivo* has been rendered moot and withdrawal thereof is respectfully requested.

Further, Applicants submit respectfully that such *ex vivo* genetic engineering is presumptively enabled inasmuch as a claim to such engineered cells has been allowed in a parent case to the present application. The present application is a continuation of 09/115,920, which has been allowed, which is a continuation of 08/751,546, now U.S. Patent No. 5,789,246. Claim 2 of U.S. Patent No. 5,789,246 recites "genetically engineered hepatocyte precursor cells obtained by culturing ... hepatocyte precursor cells capable of differentiating into hepatocytes ... to provide expanded hepatocyte precursor cells and genetically engineering the expanded hepatocyte precursor cells." Accordingly, Applicants submit respectfully that the rejection based on the alleged lack of enablement of genetically engineering cells *in vitro* has been overcome and withdrawal thereof is respectfully requested.

With respect to the allegation, made in the Final Office Action on pages 4-5, that the specification as filed does not provide any substantive guidance for demonstrating that isolated hepatocyte precursor cells will maintain a precursor-like state or will differentiate into mature

hepatocytes *in vitro* or *in vivo*, Applicants submit respectfully that, not only does the specification as filed provide literal support for the contention that hepatic precursors may maintain a precursor-like state or differentiate into mature hepatocytes under various conditions, but that such contention is presumptively true as confirmed on the record. Respectfully, Applicants turn Examiner's attention to page 13, bottom, of paper number 7, Final Office Action mailed December 4, 2000, which recites that the capability of a hepatocyte precursor cell to differentiate into a hepatocyte "is a necessary and defining characteristic of a hepatocyte precursor cell." However, as noted above, and solely to advance prosecution of the present application, and without acquiescing in the propriety of any argument that hepatic precursor cells *in vivo* do not have the necessary and defining characteristic of either maintaining a precursor state or differentiating into mature hepatocytes, Applicants have amended the claims herein to delete all references to culture of hepatocyte precursors *in vivo*.

With regard to cell culture of hepatocyte precursors and the ability of such precursors to either maintain a precursor state or differentiate into mature hepatocytes *in vitro*, Applicants submit respectfully that literal support for such cell culture is replete within the entirety of the specification as filed; see, for example, pages 6-7. Furthermore, allowed claims in the patents issued from parent applications of the present application are directed to cell cultures of hepatic precursor cells that have the capability to differentiate into mature hepatocytes (see, for example, Claim 1 of U.S. Patent No. 5,789,246); therefore, such cell culture is presumptively enabled in the present application. Accordingly, Applicants submit respectfully that U.S. Patent No. 5,789,246, by demonstrating that such cell cultures of hepatic precursor cells have the capability to differentiate into mature hepatocytes, effectively rebuts the *prima facie* case of lack of enablement.

Furthermore, the ability of liver precursor cells to differentiate is amply shown by papers in the field of the invention. Sigal *et al.* (1995)(a copy of which is attached hereto and which is also cited in the accompanying Information Disclosure Statement), transplanted precursor cells that expressed dipeptidyl peptidase IV (DPPIV) into rats that lacked this cell marker, and found expression of the marker in hepatocytes in the recipient animals. "There were numerous epithelial cells . . . with a morphology similar to adult hepatocytes. Transplanted cells were unequivocally localized by the DPPIV positivity." Page 41, paragraph four. Dabeva *et al.* (2000)(a copy of which is attached hereto) reach the same result and state that "[o]ne week after transplantation of FLEC [fetal liver epithelial cells] into the liver of normal adult rats, cells scattered throughout the parenchyma were diffusely stained for DPPIV... This suggested that the cells were not fully differentiated. Two weeks after transplantation, cells in the parenchyma . . . acquired an hepatocytic morphology with canalicular expression of DPPIV." Page 2022, paragraph one.

Accordingly, Applicants submit respectfully that the rejection based on alleged lack of enablement for demonstrating that isolated hepatocyte precursor cells will maintain a precursor like state or will differentiate into mature hepatocytes *in vitro* or *in vivo* has been overcome with respect to the *in vitro* condition and obviated with respect to the *in vivo* condition.

D. Rejection Based On The Treatment Of Any Form Of Liver Dysfunction With A Genetically Modified Hepatocyte Precursor Cell Should Be Withdrawn

The Final Office Action, from pages 5 to 7, presents a substantial discussion of alleged deficiencies in the art of gene therapy at the time of the invention. As

noted above, the Final Office Action alleges that the specification of the present application as filed is not enabling for affecting treatment of any form of liver dysfunction by genetically engineering a hepatocyte precursor cell *in vivo* or *ex vivo*, and that the specification “provides a curt description of methodology for inserting a gene of interest and administering said cell for treatment.” Applicants traverse respectfully.

Applicants submit respectfully that manipulation of cells is conventional once Applicants have taught how hepatocyte precursors are obtained. Indeed, the art is replete with examples of *in vitro* manipulation of cells, which are then administered to a patient in need thereof.

For example, Culver *et al.* (1991)(a copy of which is attached hereto and which is also cited in the accompanying Information Disclosure Statement), demonstrated that human leukocytes could be transfected *in vitro* with the human gene for adenosine deaminase (ADA) using a retroviral vector (*Id.* at page 108, paragraph 3). The autologous, genetically engineered cells were then infused into ADA-deficient children (*Id.* at page 108, paragraph 4). “Neither child has demonstrated significant side effects resulting from the infusion. . . [T]he first child *now* is making normal amounts of isohemagglutinins” (*Id.*, emphasis in the original).

Onodera *et al.* (1998)(a copy of which is attached hereto) reported successful gene therapy of a 5 year old boy with severe combined immune deficiency caused by ADA deficiency. These authors used LASN retrovirus to transfect autologous peripheral T-lymphocytes with the human ADA gene (*Id.*, page 31, paragraph 2, and page 32 paragraph 1). “No selection procedure to enrich for gene-transduced cells was performed” (*Id.*). “ADA enzyme activity, nearly undetectable in the patient’s lymphocytes before gene therapy, . . . reached 27 U on protocol day 476, which is approximately comparable to that a heterozygous carrier individual (the patient’s mother, 34.8 U)” (*Id.* at page 32, paragraph 2). “Both trials have

shown *high gene transfer efficiency, remarkable increase of the ADA enzyme activity* and eventual improvement of the immune function” (*Id.* at page 34, paragraph 0, emphasis added). “[He] has gained 3 kg in weight . . . and is attending public school” (*Id.* at page 33 paragraph 3).

Cavazzana-Calvo *et al.* (2000)(a copy of the abstract of which is attached hereto), reported gene therapy of an 11 month-old and an 8 month-old suffering from severe combined immunodeficiency-X1 disease caused by gamma-c cytokine receptor deficiency that leads to an early block in T and NK cell development. The marrow of the patients was harvested, CD34⁺ cells selected and transfected (*Id.* at 669, paragraph 2). The transfected CD34⁺ cells “were infused without prior chemoablation” (*Id.*). “Subsequently, T cell counts . . . reached values of ~2800/ul after 8 months.” (*Id.*). “After primary vaccination, *in vitro* T cell proliferative responses . . . were observed within normal range” (*Id.* at page 670, paragraph 0).

Applicants seek merely to apply standard, routine methods of cell transduction and administration, well known to those skilled in the art, using the novel isolated hepatocyte precursors of the invention.

The Final Office Action evaluated the state of the art by referring to two recent review articles, one by Verma *et al.* and one by Anderson, both of which suggest that difficulties remain with (1) efficient delivery of genes and (2) sustaining gene expression.

Applicants respectfully assert that efficiency of gene delivery is not at issue as Applicants are claiming transfection of precursor cells followed by transplanting the transfected precursors and/or their progeny. Cells bearing the gene of interest can be selected based on cotransfection with a selection marker, a conventional procedure, well known to those skilled in the art. See page 9, line 1 of the specification. Moreover, transfection with even a few copies of the relevant gene can be effective. Thus, delivery of the genes is reduced to the much simpler problem of

delivery of the cells, which can be accomplished by standard techniques, including surgery, as disclosed on page 12, line 6, for example.

Applicants also assert that sustaining gene expression is more than adequately addressed in the specification. For example, page 11, line 5, states that “a construct in which there is an additional promoter modulated by an external factor of signal can be used, *making it possible to control the level of polypeptide* being produced by the modified hepatocyte precursors, or by mature hepatocytes which have differentiated from such precursors, by providing that external factor or signal” (emphasis added). On page 12, line 23, Applicants disclose that “[o]nce in the liver [genetically engineered hepatocyte precursors] may express the gene(s) of interest and/or differentiate into mature hepatocytes which express the gene(s) of interest.” In addition, the specification teaches on page 12, line 7, that genetically engineered hepatocyte precursors or a portion of their progeny differentiates into mature hepatocytes, which *provide a continuous supply of the protein, polypeptide, hormone, enzyme, or drug encoded by the gene(s) of interest*” (emphasis added). As the articles discussed above demonstrate, similar approaches are published in the literature as providing notable successes.

Accordingly, in view of the above, Applicants submit respectfully that the rejection based on lack of enablement for affecting treatment of any form of liver dysfunction by genetically engineering a hepatocyte precursor cell *in vivo* or *ex vivo* has been overcome.

II. Rejections Under 35 U.S.C. § 112, Second Paragraph

At pages 11-13 of the Final Office Action, Claims 21-23, 25, 27, 29-36, and 39-40 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to point out particularly and distinctly claim the subject matter regarded as the invention.

Specifically, the Final Office Action alleges that: Claims 21-23, 25, 27, 29-36 and 39 are unclear and incomplete because they recite a method of treatment of liver dysfunction, however none of the claims recite steps where treatment is affected, only steps of administration; Claims 21-23, 25, 27, 29-36, 39 and 40 are vague and unclear in the recitation of ‘hepatocyte precursor’ because the specification teaches only the isolation of specific hepatocyte precursor cells, and no other type of precursors; Claims 29 and 30-33 are vague and unclear in the recitation of “expresses at least one gene of interest” because it is not clear to what “gene” the claim is referring; Claim 40 is unclear in the recitation of a “drug delivery system” because a drug encompasses many other compounds other than polypeptides which can not be synthesized by a cell and the specification only teaches the expression of potential therapeutic gene products and not administration of other types of drugs; and Claim 40 is vague and unclear in the recitation of “in a biologically significant amount.” Applicants traverse respectfully.

Respectfully, Applicants direct Examiner’s attention to the claims, as amended. Claims 21 and 22 (which has been deleted herein, and therefore the rejection thereto has been rendered moot) were amended to add a treatment step in Applicants’ Response to Final Office Action filed May 5, 2001. The claims were amended to recite “and treating liver dysfunction,” thus traversing the rejection for lack of a treatment step. Solely to advance prosecution of the present application, without acquiescing in the propriety of the rejection, and to improve the clarity of the claims, Applicants have amended Claim 21 to clarify the language related to administration and treatment so as to recite “capable of treating said liver dysfunction, and is administered to the subject.” Applicants submit respectfully that the reworded method of administration and treatment is thus clear and unambiguous. Hence, Applicants request respectfully that the aforementioned rejection of Claims 21-23, 25, 27, 29-36 and 39 be withdrawn.

Without acquiescing in the propriety of the rejections, and solely to advance prosecution of the present application, Claims 21-23, 25, 27, 29-36, 39 and 40 are amended to recite hepatocyte precursor "cell(s)," pursuant to Examiner's kind suggestion that recitation of "hepatocyte precursor cell(s)" would obviate the rejection of those claims as vague and unclear in the recitation of "hepatocyte precursor." Applicants request respectfully that the aforementioned rejection of Claims 21-23, 25, 27, 29-36 and 39-40 be withdrawn.

Without acquiescing in the propriety of the rejections, and solely to advance prosecution of the present application, Claims 29 and 30-33 are amended to recite "as the result of the genetic engineering" following "expresses at least one gene of interest." Following this amendment, Applicants submit respectfully that the claims are no longer vague and unclear in the recitation of "expresses at least one gene of interest" because it is now clear that the "gene" to which the claim refers is expressed only as a result of the genetic engineering of the hepatocyte precursor cell. Accordingly, Applicants request respectfully that the aforementioned rejection of Claims 29 and 30-33 be withdrawn.

Finally, Claim 40 is amended herein to recite a "drug delivery system for delivering an expressed therapeutic polypeptide drug or protein drug to a subject having a liver dysfunction" and to recite "express, as the result of said genetic engineering, said therapeutic polypeptide drug or protein drug in an amount effective to treat said liver dysfunction." Claim 40 had been rejected for allegedly being unclear in terms of the composition of the drug being delivered (gene product or otherwise) and allegedly being vague with regard to the meaning of "biologically significant amount." Applicants submit respectfully that the amendments made herein remove any vagueness and make the scope of the claim clear as to its metes and bounds. Accordingly, Applicants request respectfully that the aforementioned rejection of Claim 40 be withdrawn.

On the basis of the foregoing, Applicants request respectfully that the 35 U.S.C. § 112, second paragraph, rejections of Claims 21, 23, 25, 27, 29-36, and 39-40 have been overcome and withdrawal thereof is respectfully requested.

CONCLUSION

Applicants submit that the application is in condition for allowance. Favorable reconsideration, withdrawal of the rejections set forth in the above-noted Final Office Action, and an early Notice of Allowance are requested.

Applicants' undersigned attorney may be reached in our Washington, D.C. office by telephone at (202) 625-3500. All correspondence should be directed to our address given below.

AUTHORIZATION

Applicants believe there is no additional fee due in connection with this filing. However, to the extent required, the Commissioner is hereby authorized to charge any fees due in connection with this filing to Deposit Account 50-1710 or credit any overpayment to same.

Respectfully submitted,



Serge Sira, PH.D.
Registration No. 39,445
Gilberto M. Villacorta, Ph.D.
Registration No. 34,038

Patent Administrator
KATTEN MUCHIN ZAVIS ROSENMAN
525 West Monroe Street, Suite 1600
Chicago, Illinois 60661-3693
Facsimile: (312) 902-1061

Dated: December 5, 2002

EXHIBIT A

**MARKED-UP VERSION OF THE CLAIMS
U.S. PATENT APPLICATION NO. 09/534,487**

21. (Twice Amended) A method of treatment of liver dysfunction in a subject in need thereof comprising administering a genetically engineered autologous hepatocyte precursor cell, [progeny thereof, or both] wherein a hepatocyte precursor cell is removed from said subject, is genetically engineered ex vivo to be capable of treating said liver dysfunction, and is administered to the subject[and treating liver dysfunction].

22. (Canceled)

23. (Once Amended) The method of treatment of Claim 43[22] wherein the administering comprises injecting, transplanting, or grafting.

25. (Once Amended) The method of treatment of Claim 43[22] wherein the subject further comprises a liver or a spleen and the administering comprises injecting, transplanting, or grafting the genetically engineered hepatocyte precursor cell, progeny thereof, or both into the liver or the spleen of the subject.

27. (Once Amended) The method of treatment of Claim 43[26] wherein the genetic modification comprises transducing a hepatocyte precursor cell with a vector comprising a genetic material or a selectable marker.

29. (Once Amended) The method of Claim 43[22] wherein the genetically engineered hepatocyte precursor cell expresses at least one gene of interest as a result of the genetic engineering.

39. (Once Amended) The method of treatment of Claim 43[21] wherein the subject is human.

40. (Once Amended) A drug delivery system for delivering an expressed therapeutic polypeptide drug or protein drug to a subject having a liver dysfunction comprising genetically engineered hepatocyte precursor cells wherein the genetically engineered hepatocyte precursor cells express, as a result of said genetic engineering, [a] said therapeutic polypeptide drug or protein drug in an amount effective to treat said liver dysfunction[in a biologically significant amount].

EXHIBIT B

**CLAIMS WHICH WILL BE PENDING UPON ENTRY OF THE PRESENT
AMENDMENTS FILED DECEMBER 5, 2002
U.S. PATENT APPLICATION NO. 09/534,487**

21. (Twice Amended) A method of treatment of liver dysfunction in a subject in need thereof comprising administering a genetically engineered autologous hepatocyte precursor cell, wherein a hepatocyte precursor cell is removed from said subject, is genetically engineered *ex vivo* to be capable of treating said liver dysfunction, and is administered to the subject.

22. (Canceled)

23. (Once Amended) The method of treatment of Claim 43 wherein the administering comprises injecting, transplanting, or grafting.

25. (Once Amended) The method of treatment of Claim 43 wherein the subject further comprises a liver or a spleen and the administering comprises injecting, transplanting, or grafting the genetically engineered hepatocyte precursor cell, progeny thereof, or both into the liver or the spleen of the subject.

27. (Once Amended) The method of treatment of Claim 43 wherein the genetic modification comprises transducing a hepatocyte precursor cell with a vector comprising a genetic material or a selectable marker.

29. (Once Amended) The method of Claim 43 wherein the genetically engineered

hepatocyte precursor cell expresses at least one gene of interest as a result of the genetic engineering.

30. The method of Claim 29 wherein the gene of interest comprises a normal liver gene, a gene not expressed in mature normal liver cells, a gene with increased level of expression, or a combination thereof.

31. The method of Claim 29 wherein the gene of interest is incorporated into the genomic DNA of the subject.

32. The method of Claim 29 wherein the gene of interest is incorporated into the subject extrachromosomally.

33. The method of Claim 29 wherein the gene of interest comprises deoxyribonucleic acid or ribonucleic acid.

34. The method of treatment of Claim 29 wherein the gene of interest can be used to treat a viral hepatitis, correct a low density lipoprotein receptor, correct a deficiency of ornithine transcarbamylase, treat hemophilia, treat an alpha-1 anti-trypsin deficiency, treat phenylketonuria, or treat another defect in a metabolic pathway.

35. The method of treatment of Claim 29 wherein the gene of interest codes for a protein or polypeptide.

36. The method of treatment of Claim 35 wherein the protein or polypeptide is useful in prevention or therapy of an acquired or an inherited defect in liver function.

39. (Once Amended) The method of treatment of Claim 43 wherein the subject is human.

40. (Once Amended) A drug delivery system for delivering an expressed therapeutic polypeptide drug or protein drug to a subject having a liver dysfunction comprising genetically engineered hepatocyte precursor cells wherein the genetically engineered hepatocyte precursor cells express, as a result of said genetic engineering, said therapeutic polypeptide drug or protein drug in an amount effective to treat said liver dysfunction.

41. (Newly Added) A method of treatment of liver dysfunction in a human subject in need thereof comprising administering a histocompatible normal hepatocyte precursor cell, progeny thereof, or both to the human subject and treating liver dysfunction, wherein the normal hepatocyte precursor cell has been removed previously from a histocompatible donor and is capable of treating the liver dysfunction in said human subject.

42. (Newly Added) The method of Claim 41 wherein said hepatocyte precursor cell, after having been removed previously from a histocompatible donor, is further genetically engineered *ex vivo* to be capable of treating said liver dysfunction in said human subject.

43. (Newly Added) The method of Claim 21 further comprising administering to the subject progeny of said genetically engineered autologous hepatocyte precursor cell.



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EXHIBIT C

**NOTICE OF APPEAL FROM THE PRIMARY EXAMINER
TO THE BOARD OF PATENT APPEALS AND INTERFERENCES
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Application of: REID *et al.*

Application No.: 09/534,487

Group Art Unit: 1632

Filed: March 24, 2000

Examiner: Joseph T. Woitach

For: PROLIFERATION OF HEPATOCYTE
PRECURSORS

Attorney Docket No. 320018.00119

NOTICE OF APPEAL

Honorable Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants hereby appeal to the Board of Patent Appeals and Interferences from the decision dated June 5, 2002, of the Primary Examiner finally rejecting Claims 21-23, 25, 27, 29-36, and 39-40.

The items checked below are appropriate.

1. ☒ A Petition for a Three-Month Extension of Time to respond to the final rejection, together with the \$460.00 extension fee under 37 C.F.R. §1.17, is enclosed.

2. ☐ A Petition for an additional ____ month extension of time to take further action, together with the \$____ extension fee under 37 C.F.R. §1.17, was filed on ____.

3. ☐ Fee \$320.00

☒ Fee \$160.00 (Verified Statement claiming small entity status is enclosed, if not filed previously.)

☒ Enclosed

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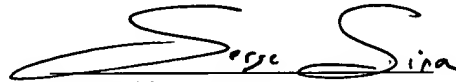
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- ☐ Not required (fee paid in prior appeal)
- ☐ Charge to Deposit Account No. 50-1710 (One additional copy of this Notice enclosed herewith)

4. ■ Any prior general authorization to charge an issue fee under 37 C.F.R. §1.18 to Deposit Account No. 50-1710 is hereby revoked. The Commissioner is hereby authorized to charge any additional fees under 37 C.F.R. §§1.16 or 1.17 which may be required during the entire pendency of this application, or to credit any overpayment, to Deposit Account No. 50-1710.

5. ■ Applicants' undersigned attorney may be reached in our Washington, D.C. office by telephone at (202) 625-3500. All correspondence should continue to be directed to our address given below.

Respectfully submitted,



Serge Sira, PH.D.
Registration No. 39,445
Gilberto M. Villacorta, Ph.D.
Registration No. 34,038

Patent Administrator
KATTEN MUCHIN ZAVIS ROSENMAN
525 West Monroe Street, Suite 1600
Chicago, IL 60661-3693
Telephone: (202) 625-3500
Facsimile: (312) 902-1061

Date: December 5, 2002



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EXHIBIT D

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: REID *et al.*

Application No.: 09/534,487

Group Art Unit: 1632

Filed: March 24, 2000

Examiner: Joseph T. Woitach

For: PROLIFERATION OF HEPATOCYTE
PRECURSORS

Attorney Docket No. 320018.00119

PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. § 1.136(a)(1)

Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants in the aforementioned matter petition to extend the time for response to the Final Office Action dated June 5, 2002, paper number 7, for three months from September 5, 2002 up to and including December 5, 2002.

Applicants' undersigned attorney may be reached in our Washington, D.C. office by telephone at (202) 625-3500. All correspondence should be directed to our address given below.

AUTHORIZATION

Please find enclosed a check for \$460.00 in payment of the extension fee. Applicants believe there is no additional fee due in connection with this filing. However, to the extent required, the Commissioner is hereby authorized to charge any fees due in connection with this filing to Deposit Account 50-1710 or credit any overpayment to same.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Serge Sira', with a horizontal line drawn underneath the name.

Serge Sira, PH.D.
Registration No. 39,445
Gilberto M. Villacorta, Ph.D.
Registration No. 34,038

Patent Administrator
KATTEN MUCHIN ZAVIS ROSENMAN
525 West Monroe Street, Suite 1600
Chicago, Illinois 60661-3693
Facsimile: (312) 902-1061

Dated: December 5, 2002



EXHIBIT E

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: REID *et al.*

Application No.: 09/534,487

Group Art Unit: 1632

Filed: March 24, 2000

Examiner: Joseph T. Woitach

For: PROLIFERATION OF HEPATOCYTE
PRECURSORS

Attorney Docket No. 320018.00119

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

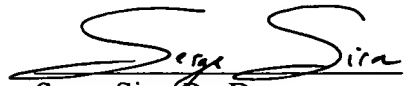
In accordance with the provisions of 37 CFR §1.56, §1.97 and §1.98, the attention of the Patent and Trademark Office is hereby directed to the documents listed on the attached form PTO-1449, cited in the attached Amendment. It is respectfully requested that the documents be expressly considered during the prosecution of the application, and that the documents be made record therein and appear among the "References Cited" on any patent to issue therefrom. The listing of any reference therein is not to be construed as an admission that any reference listed is in fact "prior art".

The relevance of each non-English language reference, if any is discussed in the present specification.

Please find enclosed a check for \$180.00 in payment of the Information Disclosure

Statement submission fee under CFR §1.97(d). The Commissioner is hereby authorized to charge any fees due in connection with this filing to Deposit Account 50-1710 or credit any overpayment to same.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Serge Sira", with a horizontal line drawn underneath the name.

Serge Sira, Ph.D.
Registration No. 39,445
Gilberto M. Villacorta, Ph.D.
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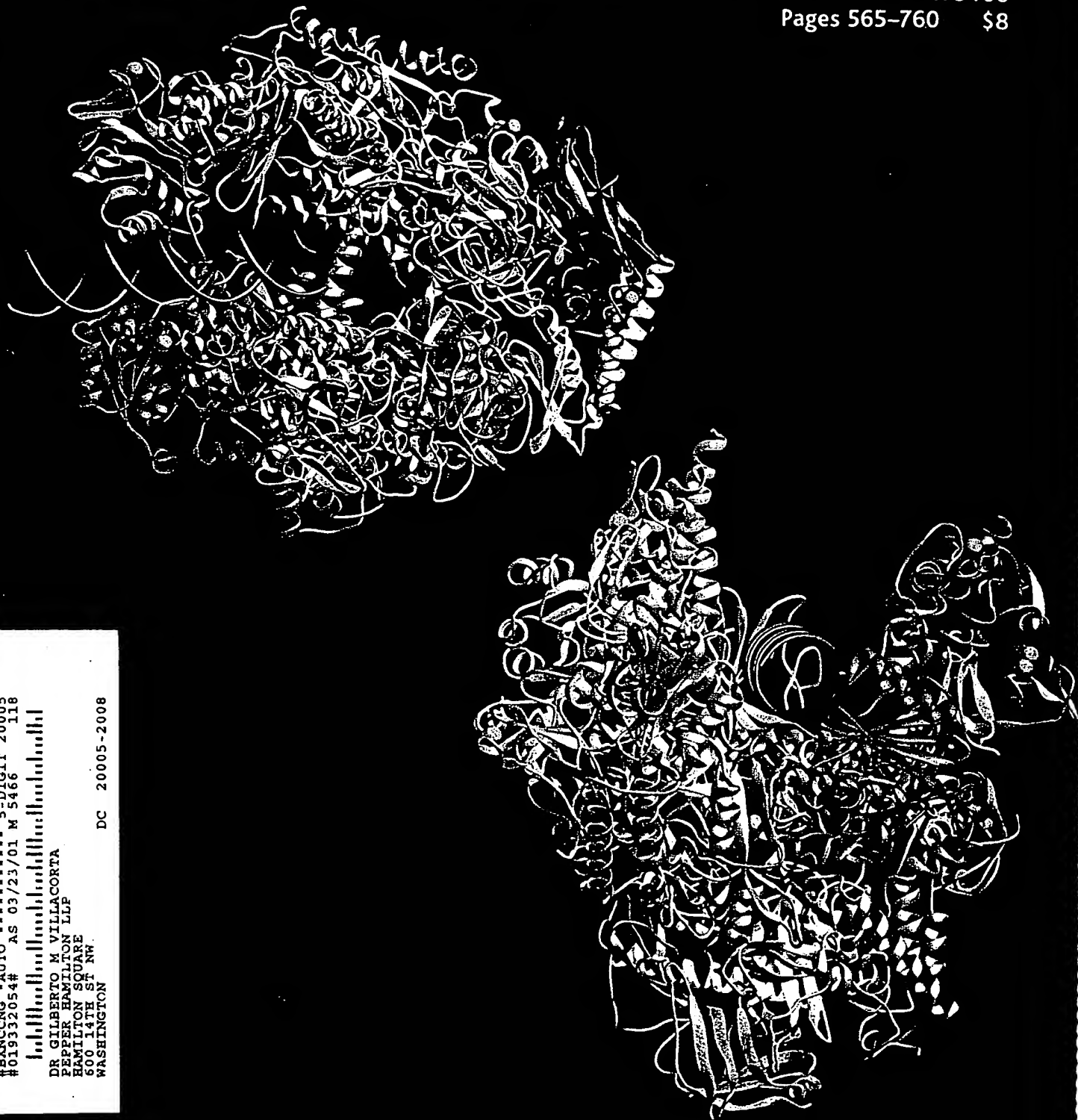
Patent Administrator
KATTEN MUCHIN ZAVIS ROSENMAN
525 West Monroe Street, Suite 1600
Chicago, Illinois 60661-3693
Facsimile: (312) 902-1061

Dated: December 5, 2002

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DR GILBERTO M VILLACORTA
PEPPER HAMILTON LLP
HAMILTON SQUARE
600 14TH ST NW
WASHINGTON

DC 20005-2008



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43. We thank P. Damiani, J. Kane, C. Delegge, K. Cuniff, C. Malcuit, and E. Milano (Advanced Cell Technology) and the staff at Trans Ova Genetics, particularly the Genetic Advancement Center Team. We also thank A. P. Soler for his help with the electron microscopy studies and K. Chapman for helpful criticism. This work was supported in part by NIH grants AG00378, AI29524, and GM56162, a grant from the National Cancer Institute of Canada, and funds from the Terry Fox Run and the Lankenau Foundation. G.M.B. is supported by the Swiss National Science Foundation.

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Gene Therapy of Human Severe Combined Immunodeficiency (SCID)-X1 Disease

Marina Cavazzana-Calvo,^{*1,2,3} Salima Hacein-Bey,^{*1,2,3}
Geneviève de Saint Basile,¹ Fabian Gross,² Eric Yvon,³
Patrick Nusbaum,² Françoise Selz,¹ Christophe Hue,^{1,2}
Stéphanie Certain,¹ Jean-Laurent Casanova,^{1,4} Philippe Bousso,⁵
Françoise Le Deist,¹ Alain Fischer^{1,2,4†}

Severe combined immunodeficiency-X1 (SCID-X1) is an X-linked inherited disorder characterized by an early block in T and natural killer (NK) lymphocyte differentiation. This block is caused by mutations of the gene encoding the γ c cytokine receptor subunit of interleukin-2, -4, -7, -9, and -15 receptors, which participates in the delivery of growth, survival, and differentiation signals to early lymphoid progenitors. After preclinical studies, a gene therapy trial for SCID-X1 was initiated, based on the use of complementary DNA containing a defective γ c Moloney retrovirus-derived vector and ex vivo infection of CD34⁺ cells. After a 10-month follow-up period, γ c transgene-expressing T and NK cells were detected in two patients. T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls. Thus, gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit.

In considering diseases that might be ameliorated by gene therapy, a setting in which a selective advantage is conferred by transgene expression, in association with long-lived transduced cells such as T lymphocytes, may prove critical. SCID-X1 offers a reliable model for gene therapy because it is a lethal condition that is, in many cases, curable by allogeneic bone marrow transplantation (1-4). It is caused by γ c cytokine receptor deficiency that leads to an early block in T and NK lymphocyte differentiation (1-3). In vitro experiments of γ c gene transfer have shown that γ c expression can be restored (5-7), as well as T and NK cell development (8-9), while the immunodeficiency of

γ c⁻ mice can be corrected by ex vivo γ c gene transfer into hematopoietic precursor cells (10, 11). Long-term expression of human γ c has also been achieved by retroviral infection of canine bone marrow (12). It has been anticipated that γ c gene transfer should confer a selective advantage to transduced lymphoid progenitor cells because, upon interaction with interleukin-7 (IL-7) and IL-15, the γ c cytokine receptor subunit transmits survival and proliferative signals to T and NK lymphocyte progenitors, respectively (2, 3). This hypothesis received further support from the observation that a spontaneously occurring γ c gene reverse mutation in a T cell precursor in one patient led to a partial, but sustained, correction of the T cell deficiency, including at least 1000 distinct T cell clones (13, 14). Spontaneous correction of the immunodeficiency has otherwise not been observed in several hundred γ c-deficient SCID patients nor in γ c⁻ mice (2-4).

Two patients, aged 11 months (P1) and 8 months (P2), with SCID-X1 met the eligibility criteria for an ex vivo γ c gene therapy trial.

SCID-X1 diagnosis was based on blood lymphocyte phenotype determination and findings of γ c gene mutations resulting either in a tail-less receptor expressed at the membrane (P1) (R289 X) or in a protein truncated from the transmembrane domain that was not expressed at cell surface (P2) (a frameshift causing deletion of exon 6) (15). After marrow harvesting and CD34⁺ cell separation, 9.8×10^6 and 4.8×10^6 CD34⁺ cells per kilogram of body weight from P1 and P2, respectively, were pre-activated, then infected daily for 3 days with the MFG γ c vector-containing supernatant (16). CD34⁺ cells (19×10^6 and 17×10^6 /kg, respectively) were infused without prior chemoablation into P1 and P2, ~20 to 40% and 36% of which expressed the γ c transgene as shown by either semiquantitative PCR analysis (P1) or immunofluorescence (P2). As early as day +15 after infusion, cells carrying the γ c transgene were detectable by PCR analysis (17) among peripheral blood mononuclear cells. The fraction of positive peripheral blood mononuclear cells increased with time (Fig. 1). T lymphocyte counts increased from day +30 in P1 (who had a low number of autologous T cells before therapy), whereas γ c-expressing T cells became detectable in the blood of P2 at day +60 (Fig. 2). Subsequently, T cell counts, including CD4⁺ and CD8⁺ subsets, increased to 1700/ μ l from day +120 to +150 and reached values of ~2800/ μ l after 8 months (Fig. 2). Transgenic γ c protein expression could not be studied on P1 cells given the presence of the endogenous tail-less protein. However, semiquantitative PCR performed at day +150 showed that a high proportion of T cells carry and express the γ c transgene (Fig. 1, A and B). Similar results were observed at day +275. Southern blot analysis of provirus integration in peripheral T cells from both patients revealed a smear indicating that multiple T cell precursors had been infected by the retroviral vector (18).

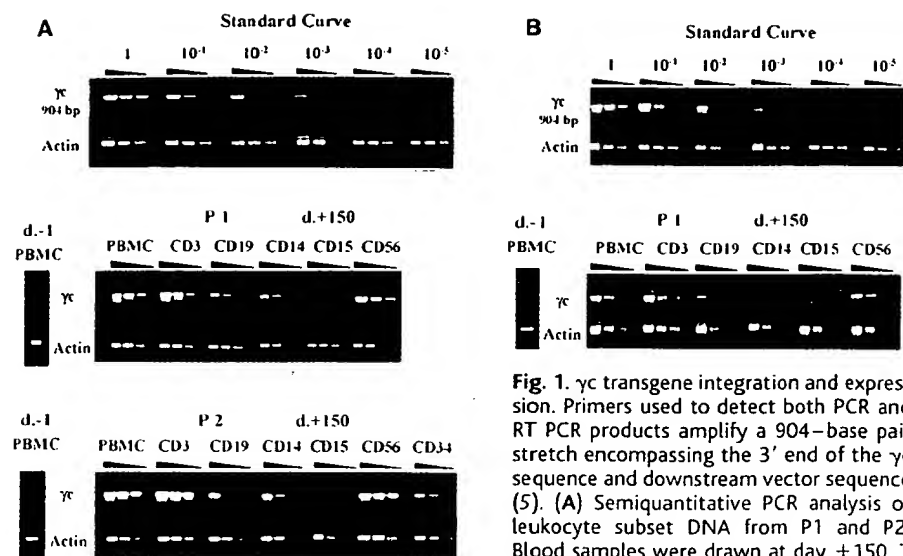
Immunofluorescence studies showed that γ c was expressed on the membrane of T cells in P2. The magnitude of expression was similar to that of control cells (Fig. 3A), as found in previous in vitro gene transfer experiments (5, 8, 9). These results indicate that sufficient transgene expression had been achieved and that γ c membrane expression is likely to be regulated by the availability of the other cytokine receptor subunits with which γ c associates (3). Both $\alpha\beta$ and $\gamma\delta$ T

¹INSERM Unit 429, ²Gene Therapy Laboratory, ³Cell Therapy Laboratory, ⁴Unité d'Immunologie et d'Hématologie Pédiatriques, Hôpital Necker, 75743 Paris Cedex 15, France. ⁵INSERM Unit 277, Institut Pasteur, 75730 Paris, France.

*These authors contributed equally to this work.

†To whom correspondence should be addressed at INSERM Unit 429, Hôpital Necker-Enfants Malades, 149 rue de Sévres, 75743 Paris Cedex 15, France. E-mail: fischer@necker.fr

REPORTS



(CD14⁺), granulocytes (CD15⁺), and NK cells (CD56⁺) as well as CD34⁺ from a bone marrow sample obtained at day +150 from P2 were isolated by a FACStar plus cell sorter (Becton Dickinson) after staining with appropriate mAbs (19). Purity was >99%. Sorted cells were analyzed for the frequency of vector-containing cells (17). Actin DNA was amplified in parallel. Samples from peripheral blood mononuclear cells (PBMC) obtained before treatment are shown as negative controls. A standard curve was constructed by diluting cells containing one copy of the MFG γc vector (5) with noninfected cells. All specimens were tested at three dilutions: 1:1, 1:20, and 1:200. (B) Semiquantitative RT-PCR analysis of leukocyte-subset RNA from P1. The same blood sample as in (A) was used. Actin cDNA was amplified in parallel as a control of RNA content. The standard curve was constructed as in (A) (17). No signal was detected in the absence of reverse transcriptase (not shown). Each specimen was diluted to 1:1, 1:500, and 1:5000.

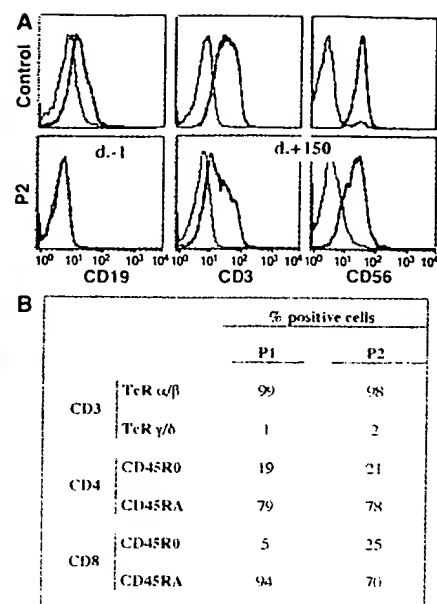


Fig. 3. γc protein expression and lymphocyte subsets. (A) γc protein detection at the surface of lymphocyte subsets from a control and from P2 obtained at day +150. γc expression on B cells from P2 after treatment was undetectable (not shown). The y axis depicts the relative cell number, and the x axis shows the logarithm of arbitrary immunofluorescence units. Thin lines are isotype controls; thick lines, staining by the anti- γc . Similar results were observed on blood samples obtained at days 275 (P1) and 240 (P2). (B) The percentage of CD45RO⁺ and CD45RA⁺ among CD4 and CD8 T cells from P1 and P2 obtained at day +275 and 240, respectively, as well as the percentage of T cells expressing either an $\alpha\beta$ TCR or a $\gamma\delta$ TCR.

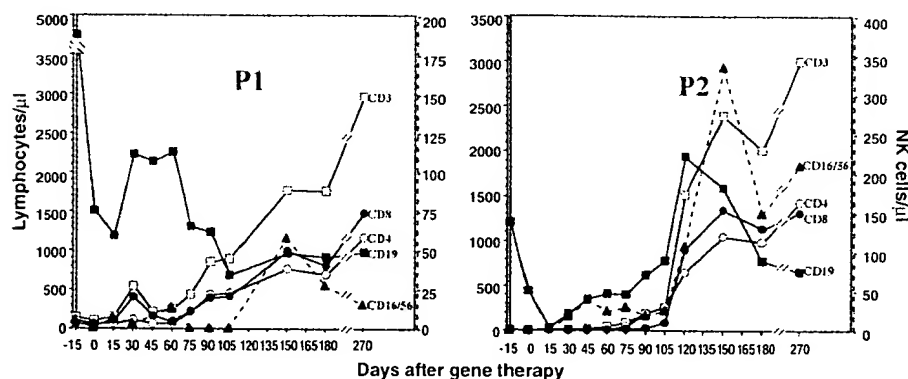


Fig. 2. Longitudinal study of lymphocyte subsets from patient 1 (P1) and patient 2 (P2). Absolute counts of T cells (CD3⁺, CD8⁺, and CD4⁺), B cells (CD19⁺), and NK cells (CD16⁺, CD56⁺) are shown as a function of time. Day 0 is the date of treatment. The scale for NK cells is on the right-hand side of each panel.

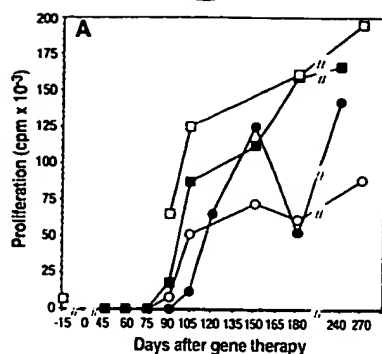
cell receptor (TCR)-expressing T cells were detected (Fig. 3B). Polyclonality and V_β TCR diversity were demonstrated by using antibodies specific for TCR V_β (19) and the immunoscope method (18, 20). In both patients, naïve CD45RA⁺ T cells were detected, accounting for a majority of the T cell subset (Fig. 3B). In both patients, T cells proliferated from day +105 in the presence of phytohemagglutinin (PHA) and antibodies to CD3 (anti-CD3). The extent of proliferation was the same as that of age-matched controls (Fig. 4A). After primary vaccination, in vitro T cell proliferative responses to tetanus toxoid (P1 and P2: 18,000 and 12,000

cpm, respectively) and polioviruses (P2: 38,000 cpm) were observed within normal range (21). P1 T cells were also found to proliferate in the presence of protein pure derivative (PPD) (12,000 cpm) as a likely consequence of bacillus Calmette-Guerin (BCG) persistence after immunization at 2 months of age in this immunocompromised child. Five months after cessation of intravenous immunoglobulin (Ig) therapy, antibodies to tetanus and diphtheria toxoids as well as to polioviruses were found in the serum of both patients, together with detectable concentrations of IgG and IgM (Fig. 4B). A normal level of IgA was also detected in the serum of

P1. As determined by semi-quantitative PCR and reverse transcriptase-PCR analysis, it was observed that in both cases, a low fraction of B cells carry and express the γc transgene (Fig. 1). It is therefore unknown whether antibody responses are provided by untransduced or the few transduced B cells. Residual persistence (<1%) of administered intravenous immunoglobulins (last given 5 months before measurement of antibody response) could, in part, also contribute. The γc -expressing NK cells were detected in the blood of P2 by day 30 (Figs. 1, 2, and 3A). These cells efficiently killed K562 cells in vitro (18). NK cells became detectable in the blood of P1 only from day +150.

As a likely consequence of development and sustained function of the immune system, clinical improvement was observed in both patients. In P2, protracted diarrhea as well as extensive graft-versus-host disease (GVHD)-like skin lesions disappeared. Both patients left protective isolation at days 90 and 95 and are now at home 11 and 10 months, respectively, after gene transfer without any treatment. Both enjoy normal growth and psychomotor development. No side effects have been noted. A similar result has since been achieved in a third patient 4 months

Fig. 4. Functional characteristics of transduced lymphocyte subsets. (A) Longitudinal follow-up of PHA (□, ■)– and anti-CD3 (○, ●)–induced proliferation of lymphocytes from P1 (open symbols) and P2 (filled symbols) (8). Background [^3H]thymidine uptake was less than 400 cpm. Positive control values are $>50 \times 10^3$ cpm. (B) Serum immunoglobulin analysis was determined by nephelometry and serum antibody by enzyme-linked immunosorbent assay after immunization (see above). Diphtheria toxoid (Dipht. tox.) was also used for immunization. The



	IgG	IgA mg/dl	IgM	Antibodies to :		
				Tet. tox.	Dipht. Tox.	Polio virus
				(iu/ml)	(iu/ml)	I II III Titer x 10 ⁴
P1 (d. + 270)	323	41	202	.53	.86	320 160 160
P2 (d. + 240)	309	0	46	.93	.63	640 640 160
Control (age matched)	420-850	16-80	40-90	>.20	>.20	>80

after gene transfer (22). These results demonstrate that in these patients, a selective advantage was conferred to T and NK lymphocyte progenitors, enabling full-blown development of mature and functioning T and NK lymphocytes (23).

These overall positive results contrast with the failure of previous attempts to perform ex vivo gene therapy in adenosine deaminase (ADA)-deficient patients (24–27). Concomitant administration of ADA enzyme to these patients is likely to have counterbalanced the potential growth advantage of the transduced cells in this setting (23). Also, advances in the methodology of gene transfer into CD34⁺ cells, i.e., the use of a fibronectin fragment (28) as well as of a cytokine combination enabling potent CD34 cell proliferation, contributed to the success of γc gene therapy.

Because γc gene transfer was achieved without any additional myeloablative or immunosuppressive therapy, these results pave the way for a possible extension of this therapeutic approach to other genetic diseases characterized by defective cell-subset generation, such as other forms of SCID (29). The kinetics of T cell development in γc gene transfer is similar to that observed in SCID patient recipients of haploidentical stem cell transplantation (4), suggesting that early progenitor cells have been infected by the MFG γc virus and effectively transduced. The hypothesis that transduced autologous T cells in P1 account for the development of the T cell compartment is unlikely because (i) the infected CD34⁺ cell population was contaminated by less than 0.1% CD3⁺ T cells; (ii) a thymic gland (27 mm by 25 mm by 25 mm at day +275) became detectable by ultrasound echography, indicative of thymopoiesis, whereas most T cells at day +275 exhibit a naïve CD45RA⁺ phenotype; and (iii) the T cell repertoire was polyclonal and diverse. In both patients, it was shown that at day +150, a fraction of bone marrow CD34⁺ cells harbored and expressed the γc transgene (Fig. 1, P2). It was not possible to determine whether more primitive cells, i.e., CD34⁺CD38[−] cells, were

transduced because of insufficient bone marrow sample. In the mouse, a common lymphoid progenitor (CLP) gives rise to the different lymphocyte populations (30). If a human counterpart of CLP exists, it would be the best candidate from among the earliest cells that were transduced ex vivo from these patients. Identification of integration sites in the various cell lineages could help determine the permissive differentiation stage. The question of the persistence of T and NK cell generation has yet to be addressed. If infected cells have no self-renewal capacity and have a short life-span, new generation of T and NK cells should cease. However, the fact that a thymic gland is still detectable 9 months after γc gene transfer suggests that thymopoiesis is still ongoing. Follow-up of the SCID-X1 patient in whom a spontaneous reversion mutation occurred in a T cell precursor (13, 14) indicates that gene transfer could be sufficient to provide a functional memory T cell pool for a number of years. This optimistic view will require careful sequential appraisal. Kohn *et al.* have previously shown that transgenes placed under the control of the long-terminal repeat (LTR) viral promoter can be silenced in quiescent T cells (31). Although the identification of silencing sequences in the MFG LTR makes this a strong possibility (31), down-regulation of γc expression has not been observed so far in these two patients, in γc -deficient mice treated by ex vivo γc gene transfer (11), or in cell lines maintained in culture over 1 year (5).

Follow-up will be required to assess the long-term effects of ex vivo γc gene transfer in CD34⁺ cells of SCID-X1 patients. To date, this methodology has resulted in the sustained correction (up to 10 months) of the SCID-X1 phenotype in two patients, including a patient in whom the mutated protein is expressed at the cell surface. It is presumed that the effect results from a strong positive selective pressure provided to the corrected lymphoid progenitors.

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last intravenous Ig injections were given at day +90 in both patients. Tet. Tox., tetanus toxoid. Isohemagglutinins to blood group A have now been detected in both patients' sera.

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15. Patient 1 had pneumocystis carinii pneumonia and had received BCG immunization. Patient 2 suffered from recurrent oral candidiasis, pneumocystis carinii infection, protracted diarrhea, failure to thrive, and GVHD-like skin lesions. Neither patient had an HLA (human leukocyte antigen)-identical sibling. Patients were placed in a sterile isolation ward and received nonabsorbable oral antibiotics and intravenous Igs every 3 weeks for 3 months. Parents gave informed consent for participation in the trial.
16. The defective MFG γc vector has been described previously (5). It was packaged in the ψcrip cell line. The MFG γc vector-containing supernatant was manufactured and provided by Genopoeitic (Lyon, France) under GMP guidelines. The vector supernatant was free of replication-competent retrovirus as determined by S+L- assay and a β -galactosidase mobilization test [R. H. Bassin, N. Tuttle, P. J. Fischinger, *J. Cancer* 6, 95 (1970); M. Printz *et al.*, *Gene Ther.* 2, 143 (1995)]. Concentration of the virus in the supernatant was 5×10^5 infectious virus particles (5). Marrow CD34⁺ cells were positively selected by an immunomagnetic procedure (CliniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). CD34 cells were cultured in gas-permeable stem cell culture (PL-2417) containers (Nexell Therapeutics, Irvine, CA), at a concentration of 0.5×10^6 cells/ml in X-vivo 10 medium (Biowhittaker, Walkersville, MD) containing 4% fetal cell serum (Stem Cell Technologies, Vancouver, Canada), stem cell factor (300 ng/ml, Amgen), polyethylene glycol-megabaryocyte differentiation factor (100 ng/ml, Amgen), IL-3 (60 ng/ml, Novartis), and Flt3-L (300 ng/ml, R&D Systems, Minneapolis, MN) for 24 hours at 37°C in 5% CO₂. Containers were precoated with the CH296 human fragment of fibronectin (50 $\mu\text{g}/\text{ml}$) (TaKaRa, Shiga, Japan). Retroviral containing supernatant was added every day for 3 days. Cells were then harvested, washed twice, and infused back into the patients.
17. For semiquantitative PCR and RT-PCR analysis, DNA was isolated from the indicated cell populations. A reference standard curve was constructed by diluting cells from a SCID-X1-derived Epstein-Barr virus (EBV)-B cell line containing one copy per cell of the MFG γc provirus (5) in uninfected cells from the same EBV-B cell line (100, 10, 1, 0.1, 0.01, and 0.001%). DNA from each sample was also quantified by actin gel amplification. MFG γc primers sequences and

Localization of a Short-Term Memory in *Drosophila*

T. Zars,^{1*} M. Fischer,^{1*} R. Schulz,² M. Heisenberg¹

Memories are thought to be due to lasting synaptic modifications in the brain. The search for memory traces has relied predominantly on determining regions that are necessary for the process. However, a more informative approach is to define the smallest sufficient set of brain structures. The *rutabaga* adenylyl cyclase, an enzyme that is ubiquitously expressed in the *Drosophila* brain and that mediates synaptic plasticity, is needed exclusively in the Kenyon cells of the mushroom bodies for a component of olfactory short-term memory. This demonstrates that synaptic plasticity in a small brain region can be sufficient for memory formation.

The localization of memory traces has occupied neuroscientists throughout this century (1). Approaches have ranged from surgical ablation to mapping localized necessary gene expression in transgenic animals (2, 3). Until recently, attempts to localize a memory trace have relied mainly on determining necessary brain regions (4). However, in a highly integrated network, other components besides the one being studied may also be necessary.

In insects, much attention has been paid to the mushroom bodies as the site for olfactory

learning (3, 5–8). In *Drosophila*, they are made up of about 2500 intrinsic neurons (Kenyon cells), receive multimodal sensory input, preferentially from the antennal lobe to the calyx, and send axon projections to the anterior brain where they bifurcate to form the α/β , α'/β' , and γ lobes (9). Noninvasive intervention techniques can provide mushroom body-less flies. In most respects, these flies show remarkably normal behavior but are deficient in olfactory learning (5). Genes important for olfactory memory have elevated expression levels in the mushroom bodies (6, 8). Additionally, the mushroom bodies are necessary for context generalization in visual learning at the flight simulator and the control of spontaneous walking activity (10, 11).

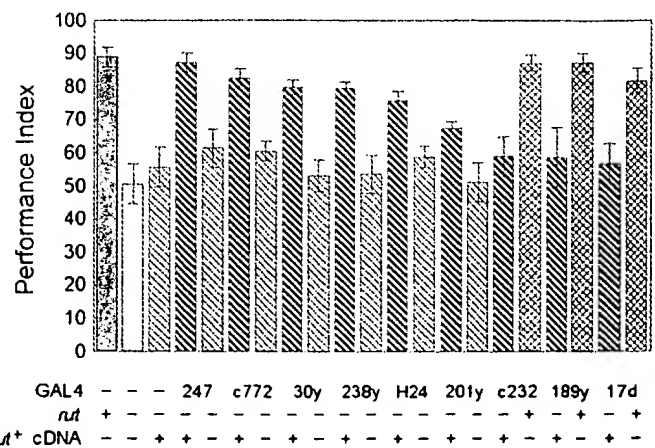
The *rutabaga* (*rut*) gene of *Drosophila* encodes a type I Ca^{2+} /calmodulin-dependent adenylyl cyclase (AC). Regulated synthesis of cyclic adenosine 3',5'-monophosphate by

¹Theodor Boveri Institut für Biowissenschaften, Lehrstuhl für Genetik, (Biozentrum) Am Hubland, D97074, Würzburg, Germany. ²Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: zars@biozentrum.uni-wuerzburg.de

Fig. 1. The *rut* mutant defect in olfactory short-term memory can be rescued with a *rut*⁺ cDNA in several GAL4 enhancer trap lines. Memory was measured about 2 min after classical conditioning (17). Performance indices (PIs) of *rut* mutant flies (white bar) and *rut* mutant flies with either a P[UAS_{GAL4}-*rut*⁺] or GAL4 enhancer trap element (thin diagonal striped bars) were significantly different from wild-type flies (dark gray bar; P 's < 0.0005). There was no significant difference between *rut* mutant flies' PIs rescued with GAL4 enhancer trap elements 247, c772, 30y, 238y, and H24 and the P[UAS_{GAL4}-*rut*⁺] compared with wild-type flies (dark gray and thick diagonal striped bars, respectively; P 's > 0.05). Mutant *rut* flies' performance was rescued with GAL4 element 201y and the P[UAS_{GAL4}-*rut*⁺] (P < 0.05) but was also significantly lower than the performance of wild-type flies (P < 0.005). GAL4 enhancer trap lines c232, 189y, and 17d with a P[UAS_{GAL4}-*rut*⁺] did not rescue the *rut* mutation (P 's > 0.05). Wild-type flies heterozygous for GAL4 enhancer trap elements c232, 189y, and 17d were not significantly different from wild-type flies (dark gray and cross-hatched bars; P > 0.05). Bars represent mean PIs; errors are SEMs; n = 6 for all genotypes.



actin primers sequences are available on request. DNA was amplified in a 50 μ l of PCR reaction mixture by using 30 cycles at an annealing temperature of 60°C for γ c primers and 68°C for actin primers. A sample of the amplified product was separated on a 1% agarose gel and analyzed by ethidium bromide staining. RNA was prepared with the RNA easy kit (Qiagen) and was reverse-transcribed with the Superscript Preamplification System (Gibco-BRL). γ c proviral and β -actin cDNA amplification were performed as described above. Quantification of expression was made by comparison with RNA isolated from the same standard curve of diluted cells.

18. M. Cavazzana-Calvo et al., data not shown.
19. The following monoclonal antibodies (mAbs) were used in immunofluorescence studies: anti- γ c chain: Tugh 4 (rat IgG2, Pharmingen, San Diego, CA); anti-CD3: Leu 4 (IgG2a, Becton Dickinson, San Diego, CA); anti-CD4: Leu3a (IgG1, Becton Dickinson); anti-CD8: Leu 2a (IgG1, Becton Dickinson); anti-CD19: J4 119 (IgG1 Immunotech, Marseille, France); anti-CD14: Leu M3 (Becton Dickinson); anti-CD16: 3C8 (IgG1, Immunotech); anti-CD56: MY31 (IgG1, Becton Dickinson); anti-CD15 (IgM, Pharmingen); anti-TCR α : BMA031 (IgG1, Immunotech); anti-TCR γ : IMM515 (IgG1, Immunotech); anti-CD45RO: UCHL1 (IgG2a, Immunotech); anti-CD45RA: 2H4 (IgG1, Coulter Clone, Margency, France); anti-CD34: HPCA-2 (IgG1, Becton Dickinson); anti-TCR V β 2: MPB205 (IgG1, Immunotech); anti-TCR V β 3: CH92 (IgM, Immunotech); anti-TCR V β 5.1: IMM157 (IgG2a, Immunotech); anti-TCR V β 5.2: 36213 (IgG1, Immunotech); anti-TCR V β 5.3: 3D11 (IgG1, Immunotech); anti-TCR V β 8: 56C5.2 (IgG2a, Immunotech); anti-TCR V β 9: FIN9 (IgG2a, Immunotech); anti-TCR V β 13.1: IMM222 (IgG2, Immunotech); anti-TCR V β 13.6: JU74.3 (IgG1, Immunotech); anti-TCR V β 14: CA51.13 (IgG1, Immunotech); anti-TCR V β 17: E17.5F3.15.13 (IgG1, Immunotech); anti-TCR V β 21.3: IG125 (IgG2, Immunotech). Fluorescence staining was done with phycoerythrin- or fluorescein isothiocyanate-conjugated mAbs. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson).
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MEETING PRESENTATION

Lymphocyte Gene Therapy

KENNETH W. CULVER, W. FRENCH ANDERSON, and R. MICHAEL BLAESE

ABSTRACT

Genetically corrected T cells are currently under investigation as a treatment for severe combined immunodeficiency disease resulting from a lack of adenosine deaminase (ADA). Monthly injections of these ADA-corrected T cells have resulted in measurable ADA activity in the peripheral blood and the *in vivo* production of antibody to blood group antigen. Genetically corrected T cells appear to be clinically valuable vehicles for gene therapy.

INTRODUCTION

ADENOSINE DEAMINASE (ADA) deficiency is a rare, often fatal autosomal recessive disorder that occurs in less than 1 in 100,000 births (Giblett *et al.*, 1972). An absence of intracellular ADA activity results in a profound immunodeficiency disease, called severe combined immunodeficiency (SCID). ADA(-) SCID accounts for approximately 25% of all cases of SCID. ADA is an enzyme in the purine salvage pathway. Adenosine (Ado) or deoxyadenosine (dAdo) is irreversibly deaminated by ADA to inosine or deoxyinosine, respectively. Adenosine and deoxyadenosine are phosphorylated by a series of kinases ultimately to yield the nucleosides ATP and dATP. These serve as substrates for nucleic acid synthesis or may be used in cellular metabolism. Immature intrathymic lymphocytes are especially rich in adenosine kinases and, in the absence of ADA, tend rapidly to convert Ado and dAdo to their triphosphate forms. dATP is thought to be a primary mediator of lymphocyte toxicity when present in excessive levels. As a result, these children have severe lymphopenia and fail to mount effective cellular and humoral immune responses. Prior to bone marrow transplantation, these children usually died before 2 years of age.

The treatment of choice is a HLA-matched bone marrow transplant that has a cure rate of >70%. Unfortunately, only about 30% of children with SCID will have a matched donor. For children with SCID and no HLA-identical sibling, the results from T-cell-depleted, haploidentical (*i.e.*, parents as donors) marrow transplants without cytoreduction have been less successful for ADA(-)SCID than ADA(+)SCID, with durable engraftment rates of 40% versus 60%, respectively (O'Reilly *et al.*, 1989). The difference may be due to an

increased natural killer cell activity commonly found in children with ADA(-)SCID.

ADA deficiency is a clinically heterogeneous disorder, with the spectrum ranging from severe immunodeficiency evident in the first days of life to the appearance of significant infections occurring only in later childhood (Levy *et al.*, 1988). Children who have demonstrated less severe clinical disease have generally been considered candidates for enzyme replacement therapy or gene therapy as opposed to marrow transplantation with ablation. Enzyme replacement therapy has shown some benefit because deoxyadenosine is freely diffusible between the intracellular and extracellular space. Increased levels of ADA in the plasma degrade deoxyadenosine locally and thus establish a concentration gradient leading to a flow of the accumulated deoxyadenosine from intracellular sites to the plasma where it is also degraded.

Some ADA-deficient children are currently treated with injections of a drug called Adagen (PEG-ADA) (Hershfield *et al.*, 1987). PEG-ADA is bovine ADA conjugated to polyethylene glycol, which is an inert substance that protects the ADA enzyme from catabolism thus allowing it to function for days in the blood, compared to minutes without the PEG. PEG-ADA treatment has been used since 1985 with a variable degree of immunologic enhancement in the approximately 20 children who have been treated. Unfortunately, PEG-ADA does not usually result in complete restoration of normal immune function. Therefore, we have pursued the use of gene therapy as a means to correct the fundamental problem, a defective ADA gene.

Ideally, ADA(-)SCID would be treated with the insertion of a functioning ADA gene into autologous bone marrow totipotent stem cells resulting in the continual production of ADA(+)

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lymphocytes. Many laboratories have worked for years to develop bone marrow gene therapy. Unfortunately, bone marrow gene transfer experiments in large animals (monkeys, sheep, and dogs) have resulted in low-level, transient expression of the transferred genes in peripheral blood lymphocytes (PBL) (Williams, 1990). As a result, we have developed the use of T lymphocytes as vehicles for gene therapy (Culver *et al.*, 1988).

T cells are particularly good candidates for ADA(-)SCID because engraftment of donor T cells alone following a nonimmunosuppressed bone marrow transplant results in cure of the combined immunodeficiency disease (Keever *et al.*, 1988). Even though the child remains ADA(-) throughout its body, these ADA(+) cells can survive and function, suggesting that cells containing functional ADA activity have a selective growth advantage *in vivo*. Following extensive experimentation *in vitro* with human ADA(-) cells (Culver *et al.*, 1991a), *in vivo* studies in murine and Rhesus animal models (Culver *et al.*, 1991b,c), and an indepth review by a variety of regulatory committees (Blaese *et al.*, 1990), we began our clinical trial on September 14, 1990.

MATERIALS AND METHODS

Subjects

Children with ADA(-)SCID who have been treated with Pegadamas for a minimum of 9 months and have not achieved full immune reconstitution.

Gene transfer and the growth of autologous T cells

Gene transfer utilizes genetically altered, defective mouse viruses called retroviral vectors (Gilboa *et al.*, 1986). Retroviral-mediated gene transfer requires dividing cells for stable integration and has an efficiency of 10–15% in cultured human T lymphocytes. To transfer the ADA gene, the child's blood is removed from a vein and the white blood cells are separated from the red blood cells using an apheresis procedure. The white blood cells are placed in culture with OKT3 monoclonal antibody (10 ng/ml) and recombinant interleukin-2 (rIL-2) (1,000 U/ml, Cetus). After 18 hr, the LASN retroviral vector containing a normal human ADA gene, engineered by Dr. A. Dusty Miller at the Fred Hutchinson Cancer Center (Seattle, WA) and supplied by Genetic Therapy, Inc. (Gaithersburg, MD), is mixed into the culture medium. The vector binds to the surface of the T lymphocytes, enters the cell and inserts the human ADA gene randomly into the chromosome. The human ADA gene is now stably integrated and will remain in place and be passed to all of the daughter cells as the cells grow in number. The cells are expanded in culture for a total of 9–10 days before reinfusion IV.

RESULTS

Two children have begun on infusions of ADA gene-corrected autologous T lymphocytes concurrent with PEG-ADA treatment (Fig. 1). On a monthly basis, the children undergo leukapheresis. The mononuclear cells are then placed in culture

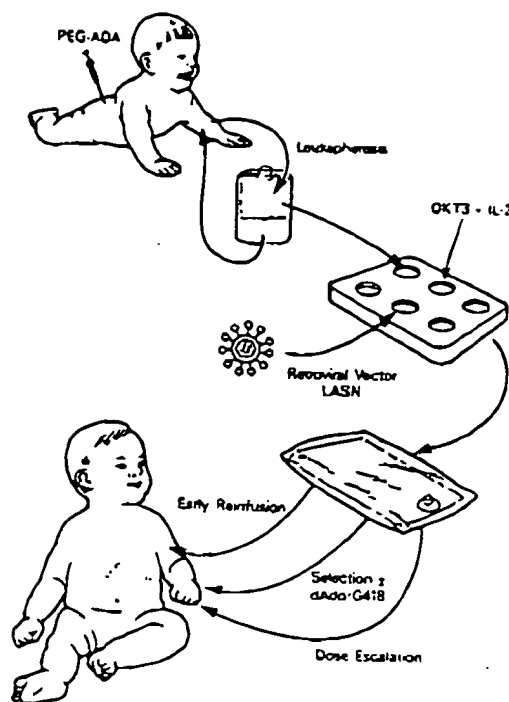


FIG. 1. Schematic of the human ADA gene therapy protocol.

in 24-well plates with the monoclonal antibody OKT3 and rIL-2, which induces vigorous T-cell proliferation. Beginning on the second day, the vector LASN is added twice daily for 3 days. The cells continue to expand in number, are transferred into gas-permeable cell culture bags, and are then reinfused after 9–11 days in culture. Once the safety of the procedure has been established, we may preselect for gene-containing cells by growth in dAdo or G418 (compounds that will kill cells without a LASN vector). With time, we plan to escalate the number of infused cells to maximize the opportunity for immune recovery.

Neither child has demonstrated any significant side effects resulting from the infusion of as many as 7×10^8 cells/kg. Evaluation of the first child has noted selective persistence of the ADA gene-corrected T cells with continued ADA protein production in cells regrown from the blood (Table 1). PBL containing the LASN vector have a selective growth advantage compared to cells without the vector *in vitro* as the ADA activity increased from 3.0 to 43.3 units of activity during 3 weeks in culture. In addition, the first child now is making normal amounts of isohemagglutinins (antibodies to blood group antigens), an immune function that was not present on PEG-ADA alone. The second child has received only two infusions to date.

DISCUSSION

Gene therapy has the potential to alter the course of human disease dramatically. Our current technology is limited by not

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TABLE 1. RECOVERY OF ADA GENE-TRANSDUCE
T CELLS FROM A SCID PATIENT

Cell population tested	ADA concentration (units)
Pretreatment PBL	1.3 ^a
PBL 3 weeks after second infusion	3.0
T cells cultured 3 weeks	43.3
Normal PBL	40-90

^a ADA enzymatic activity is expressed as nmol/min · 10⁸ cells.

only the availability of the normal genes, but a satisfactory *in vivo* delivery system. T lymphocytes are currently the best cellular delivery vehicles for human gene therapy. They can be easily transduced with retroviral vectors and easily manipulated *in vitro* and *in vivo* with growth factors and antigen-specific stimulation. Both human and animal experiments have shown prolonged survival of the gene-altered T cells with continued expression of the vector genes (Culver *et al.*, 1991b,c). Now, we have evidence that the reinfusion of ADA gene-corrected cells can provide T-helper cell function for an antibody response not seen with PEG-ADA alone. This finding is consistent with *in vitro* and *in vivo* experiments that strongly suggest that intracellular correction of ADA(-)SCID is superior to extracellular enzyme replacement alone (Ferrari *et al.*, 1991). Tumor-specific T cells, genetically altered with cytokine genes, are now beginning clinical trials.

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Address reprint requests to:

Dr. R. Michael Blaese
Metabolism Branch National Institute of Health
Building 10, Room 6B05
9000 Rockville Pike
Bethesda, MD 20892

Proliferation and Differentiation of Fetal Liver Epithelial Progenitor Cells after Transplantation into Adult Rat Liver

Mariana D. Dabeva,*†‡ Petko M. Petkov,*†‡
Jaswinder Sandhu,*†‡ Ran Oren,§ Ezio Laconi,**
Ethel Hurston,*†‡ and David A. Shafritz*†§§

the Marion Bessin Liver Research Center, the Division of Gastroenterology, Hepatology and Nutrition,† and the Departments of Medicine,‡ Cell Biology,§ and Pathology,¶ Albert Einstein College of Medicine, Bronx, New York; the Department of Gastroenterology,|| Tel Aviv Sourasky Medical Center, Tel Aviv, Israel; and the Istituto di Patologia Sperimentale,** Ospedale Oncologico "A Businco", University of Cagliari, Cagliari, Italy

To identify cells that have the ability to proliferate and differentiate into all epithelial components of the liver lobule, we isolated fetal liver epithelial cells (FLEC) from ED 14 Fischer (F) 344 rats and transplanted these cells in conjunction with two-thirds partial hepatectomy into the liver of normal and retorsine (Rs) treated syngeneic dipeptidyl peptidase IV mutant (DPPIV⁻) F344 rats. Using dual label immunohistochemistry/*in situ* hybridization, three subpopulations of FLEC were identified: cells expressing both α -fetoprotein (AFP) and albumin, but not CK-19; cells expressing CK-19, but not AFP or albumin, and cells expressing AFP, albumin, and cytokeratins-19 (CK-19). Proliferation, differentiation, and expansion of transplanted FLEC differed significantly in the two models. In normal liver, 1 to 2 weeks after transplantation, mainly cells with a single phenotype, hepatocytic (expressing AFP and albumin) or bile ductular (expressing only CK-19), had proliferated. In Rs-treated rats, in which the proliferative capacity of endogenous hepatocytes is impaired, transplanted cells showed mainly a dual phenotype (expressing both AFP/albumin and CK-19). One month after transplantation, DPPIV⁻ FLEC engrafted into the parenchyma exhibited an hepatocytic phenotype and generated new hepatic cord structures. FLEC, localized in the vicinity of bile ducts, exhibited a biliary epithelial phenotype and formed new bile duct structures or were incorporated into pre-existing bile ducts. In the absence of a proliferative stimulus, ED 14 FLEC did not proliferate or differentiate. Our results demonstrate that 14-day fetal liver contains lineage committed (unipotential) and uncommitted (bipotential) progenitor cells exerting different repopulating capacities,

which are affected by the proliferative status of the recipient liver and the host site within the liver where the transplanted cells become engrafted. These findings have important implications in future studies directed toward liver repopulation and *ex vivo* gene therapy. (*Am J Pathol* 2000, 156:2017-2031)

The liver originates from the gut endoderm. On embryonic day (ED) 8.5 in the mouse and 1 day later in the rat, primitive epithelial cells of the foregut contact the cardiac mesoderm and form the liver diverticulum.¹⁻⁶ These cells proliferate extensively, invade the septum transversum, begin to differentiate, and, on ED 9.5 in mice and ED 10.5 in rats, acquire the morphological appearance of immature liver epithelial cells (hepatoblasts), expressing first α -fetoprotein (AFP) and then albumin.^{6,7} Following the expression of these and other hepatic markers, including also cytokeratins (CKs), most authors conclude that hepatoblasts are bipotential cells, capable of differentiating along the hepatocytic or bile duct epithelial cell lineage.⁷⁻¹¹ At ED 15-16, the rat liver already contains committed immature hepatocytes and bile duct epithelial cells.^{7,8,12,13} In both rats and humans, embryonic hepatoblasts in large vascular spaces also form primitive ductal structures, which ultimately give rise to the intrahepatic bile ducts.¹⁴⁻¹⁶

A number of transcription, signaling, and growth factors have been identified that play an essential role in gut endoderm differentiation and fetal liver development. These include factors that bind to the GATA DNA sequence motif (GATA), signal transducers and activators of transcription (STATs), hepatocyte nuclear factors (HNF)-3- α and - β , HNF-4, HNF-6, and certain fibroblast growth factors (FGFs).¹⁷⁻²⁵ However, the mechanisms by which primitive pluripotential endodermal cells undergo hepatic specification and how bipotential hepatoblasts differentiate further into hepatocytes and bile duct epithelium remain largely unknown.

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Address reprint requests to David A. Shafritz, M.D., Liver Research Center, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. E-mail: shafritz@aecom.yu.edu.

Studies in the adult liver have also provided strong evidence for the existence of putative liver stem cells, ie, undifferentiated liver epithelial cells that can be activated to proliferate and differentiate into hepatocytes or bile duct epithelial cells.²⁶⁻²⁸ These cells are thought to reside within or adjacent to the canals of Hering. Unlike stem cells in other tissues, such as bone marrow, skin, and intestine, which undergo continuous renewal, liver stem-like cells are facultative; they comprise a quiescent compartment of dormant cells that is activated only if the regenerative capacity of hepatocytes is impaired. Attempts have been made to identify their counterpart in fetal liver,^{7,28-31} and it has been suggested that the dormant stem-like cells originate most probably from bipotential fetal liver epithelial progenitor cells.^{28,32,33}

To explore the ability of fetal liver epithelial progenitor cells (FLEC) to proliferate and differentiate into hepatocytes (Hc) and bile duct epithelial cells (BDEC) and become incorporated into structural components of the liver lobule, we have used a cell transplantation approach to monitor the fate of these cells under different experimental conditions. Cells were transplanted into the liver of a syngeneic mutant Fischer 344 (F344) rat strain, deficient in the exopeptidase dipeptidyl-peptidase IV (DPPIV).³⁴ Because this enzyme is expressed in both Hc and BDEC, the genetically DPPIV-deficient F344 rat is an excellent model to follow the proliferation, lobular distribution, and morphological appearance of transplanted wild-type (DPPIV⁺) hepatic cells.³⁴⁻³⁸

When normal liver is subjected to partial hepatectomy (PH), liver regeneration occurs through proliferation of pre-existing mature hepatocytes.³⁹⁻⁴⁰ However, when rats are treated with retrorsine (Rs), this pyrrolizidine alkaloid is taken up by hepatocytes and metabolized to a bioactive form, which alkylates cellular DNA. This interferes with cell cycle progression and leads to inability of hepatocytes to proliferate.⁴¹⁻⁴³ In the present study, we used both normal and Rs-treated DPPIV⁻ rats to follow the proliferation, lineage progression, and differentiation of transplanted ED 14 FLEC cells. This was evaluated by their morphological appearance, histochemical expression of DPPIV, and expression of markers specific for Hc or BDEC, using dual label immunohistochemistry and *in situ* hybridization (ISH). Our results demonstrate that FLEC are a heterogeneous population of cells with a single or dual phenotype (unipotential or bipotential) and that their lineage commitment and proliferative activity varies depending on the engraftment site and functional status of the host liver.

Materials and Methods

Materials

Rs and diaminobenzidine (DAB) were purchased from Sigma Chemical (St. Louis, MO). The Vectastain Elite ABC kit was from Vector Laboratories (Burlingame, CA). Rabbit anti-rat red blood cells IgG was from Rockland (Gilbertsville, PA). Radioactive ³⁵S-UTP (SJ603) and CK-19 antibody (RPN 1165) were obtained from Amer-

sham Life Science Products (Arlington Heights, IL). CK-14 antibody (NCL-LL002) was from Novocastra Laboratories (United States distributor, Vector Laboratories). OV-6 monoclonal antibody was a generous gift from Dr. S. Sell (Albany Medical College, Albany, NY). Digoxigenin RNA labeling mix and anti-digoxigenin-POD. Fab fragments were from Boehringer Mannheim (Indianapolis, IN). Autoradiographic emulsion, type NBT2, was purchased from Eastman Kodak Company (New Haven, CT). Dr. N. Fausto (University of Washington, Seattle, WA) kindly provided plasmid BAF700, used for synthesis of the fetal form of AFP mRNA riboprobe.

Animals and Animal Treatment

Normal Fischer rats (F344) were purchased from Charles River Laboratories (Wilmington, MA). Mutant DPPIV-deficient (DPPIV⁻) F344 rats were obtained from the Special Animal Core Facility of the Liver Research Center, Albert Einstein College of Medicine. All studies with animals were conducted under protocols approved by the Animal Care Use Committee of the Albert Einstein College of Medicine and were in accordance with National Institutes of Health guidelines. Rs treatment of the animals was as described previously.³⁸ In all experiments, cell transplantation recipients were female DPPIV⁻ F344 rats. For experiments in which cell transplantation recipients were treated with retrorsine, rats weighing 90 to 100 g were given two intraperitoneal injections of Rs, 2 weeks apart, each of 30 mg/kg body weight. One month after the second injection, animals were subjected either to two-thirds PH and transplantation or to transplantation without PH. For stimulation of cell proliferation with triiodothyronine (T3), 1 day before cell transplantation and every week thereafter, animals received subcutaneous injections of T3 (Sigma) at a dose of 400 µg/100 g body wt, for a total of four T3 injections.

Isolation of FLEC

Fourteen-day FLEC from DPPIV⁻ animals were isolated by a modification of the procedure of Sigal et al.¹² In brief, fetal livers were placed in ice-cold modified Hanks' balanced salt solution (HBSS; Gibco BRL, Grand Island, NY) without Ca²⁺, containing 0.8 mmol/L MgCl₂ and 20 mmol/L HEPES, pH 7.4, and then triturated gently several times in modified HBSS containing 1 mmol/L EGTA. After centrifugation for 5 minutes at 450 × g at 4°C, the pellet was suspended in modified HBSS containing 0.2% collagenase, 0.07% DNase, and 1 mmol/L CaCl₂. Digestion was carried out for 15 minutes at 37°C, with gentle trituration every 5 minutes. The reaction was stopped by adding an equal volume of modified HBSS-containing 1 mmol/L EGTA and fetal bovine serum at a final concentration of 10%. The cell suspension was filtered through a 45-µm nylon mesh, and cells were collected by centrifugation as above. The cell pellet was washed twice with modified HBSS/0.1% bovine serum albumin, centrifuged, and suspended at a concentration of 10⁷ cells/ml. The cell suspension was subjected to two rounds of panning

with rabbit IgG against rat red blood cells (Rockland), as described.¹²

Transplantation of FLEC

A total of 1.5 to 3.0×10^6 cells in a volume of 0.5 ml (of which approximately 15% or 2.25 to 4.50×10^5 cells were judged to be FLEC by detection of AFP mRNA using ISH of small aliquots fixed to cytospin slides) were infused into the liver through the portal vein immediately after two-thirds PH. Control animals received only cell transplantation. Four to five animals, including control animals, were used for each time point. The proliferation and differentiation of FLEC in the liver of the recipients was analyzed 1, 2, and 4 weeks after cell transplantation. The livers of T3-treated animals were analyzed 4 weeks after cell transplantation.

Histochemical Detection of DPPIV, γ -Glutamyl Transpeptidase (γ -GT), and Glucose-6 Phosphatase (G6-P)

To detect DPPIV⁺ transplanted cells in the liver of DPPIV⁺ F344 rats, histochemical staining was carried out as described previously.³⁷ γ -GT was detected by the method of Rutenburg et al.⁴⁴ as described previously⁴⁵ and G-6P by the method of Teusch,⁴⁶ with modifications described previously.⁴⁷

Immunohistochemical Detection of CK-19, CK-14, and Oval Cell (OV)-6 Antigen

Immunohistochemical detection was performed after 10 minutes' fixation in cold 4% paraformaldehyde (PFA) prepared in phosphate buffered saline (PBS). The slides were washed in PBS and then in PBS/0.1% Triton X-100. Endogenous peroxidase was blocked for 5 minutes with 5 mmol/L periodic acid, and the sections were washed for 30 minutes with 3 mmol/L sodium borohydride in PBS. Further blocking was performed according to instructions in the Vectastain ABC Elite kit (including biotin/avidin blocking). CK-19 antibody (RPN 1165) at a dilution of 1:10, CK-14 antibody (NCL-LL002) at a dilution of 1:20, and OV-6 antibody at a dilution of 1:100 were applied for 3 hours at room temperature. Biotinylated anti-mouse IgG (BA-2001) was used as a secondary antibody in combination with the Vectastain ABC kit. Peroxidase activity was developed by diaminobenzidine (DAB) staining.

ISH and Dual Immunohistochemistry/ISH Labeling

ISH was conducted on frozen sections as described previously.⁴⁵ ISH of cells collected on plus charged slides (Fisher Scientific, Springfield, NJ) by the cytospin method, including several additional steps before acetylation. In brief, after fixation, washing, and dehydration, the slides were rehydrated for 10 minutes in PBS/5

mmol/L $MgCl_2$ and permeabilized for 20 minutes in 0.1% Triton X-100, prepared in the same buffer. The slides were then washed for 5 minutes with the same buffer, treated for 5 minutes at room temperature with 5 μ g/ml of Proteinase K (in 0.1 mol/L Tris/HCl, pH 8, and 5 mmol/L EDTA), washed for 3 minutes with 0.2% glycine, fixed again for 5 minutes in PFA, washed with buffer, and acetylated.

Dual ISH labeling was performed with ³⁵S-labeled rat albumin antisense riboprobe and digoxigenin-labeled AFP antisense riboprobe. For detection of digoxigenin-labeled RNA hybrids, anti-digoxigenin POD (Fab fragment) was applied and peroxidase activity revealed by DAB.⁴⁵ The slides were dehydrated, dipped in autoradiographic emulsion (NBT2), and exposed for 1 week to detect autoradiographic grains representing albumin mRNA.

For combined (dual) immunohistochemistry and ISH labeling, frozen sections were first processed with CK-19 antibody, peroxidase activity developed with DAB, and ISH was then performed with ³⁵S-labeled AFP or albumin antisense riboprobe as described previously.⁴⁷ After washings and dehydration, the slides were exposed with autoradiographic emulsion for 1 to 3 days and stained with hematoxylin.

Screening for Y Chromosome Marker Sry in Female Rats Transplanted with FLEC

This procedure followed the protocol described by An et al.⁴⁸ First, a Sry fragment was amplified from rat genomic DNA by polymerase chain reaction, using primers homologous to the mouse sry gene. The amplified product of 459 bp was cloned into pGEM T-Easy vector (Promega, Madison, WI). Screening for Y-chromosome DNA in female livers transplanted with FLEC was carried out using rat Sry primers as follows: Primer 1, Rat Sry (5'-CATC-GAAGGGTTAAAGTGCCA-3') and primer 2, Rat Sry-R (5'-ATAGTGTGTAGGTTGTTGTCC-3'). These primers amplify a stretch of 104 bp nested within the 459-bp fragment. Rat liver DNA from the recipient livers and control male and female rats was purified using the DNEasy kit (Qiagen Inc., Valencia, CA). Serial dilution of the DNA samples beginning with 50 ng of DNA per reaction were prepared in a 50- μ l reaction mixture containing 2.5 mmol/L $MgCl_2$, 0.4 μ mol/L of each primer, 0.2 mmol/L of each dNTP, and 1 unit of Platinum-Taq polymerase (Gibco BRL, Grand Island, NY). Amplification conditions included a 3-minute incubation at 94°C, followed by 32 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C, and a final termination step of 7 minutes at 72°C. The product was resolved on a 2% agarose gel.

Results

Characteristics of Isolated FLEC

Rat liver at ED 14 contains immature epithelial cells and a large number of hematopoietic cells at different stages of

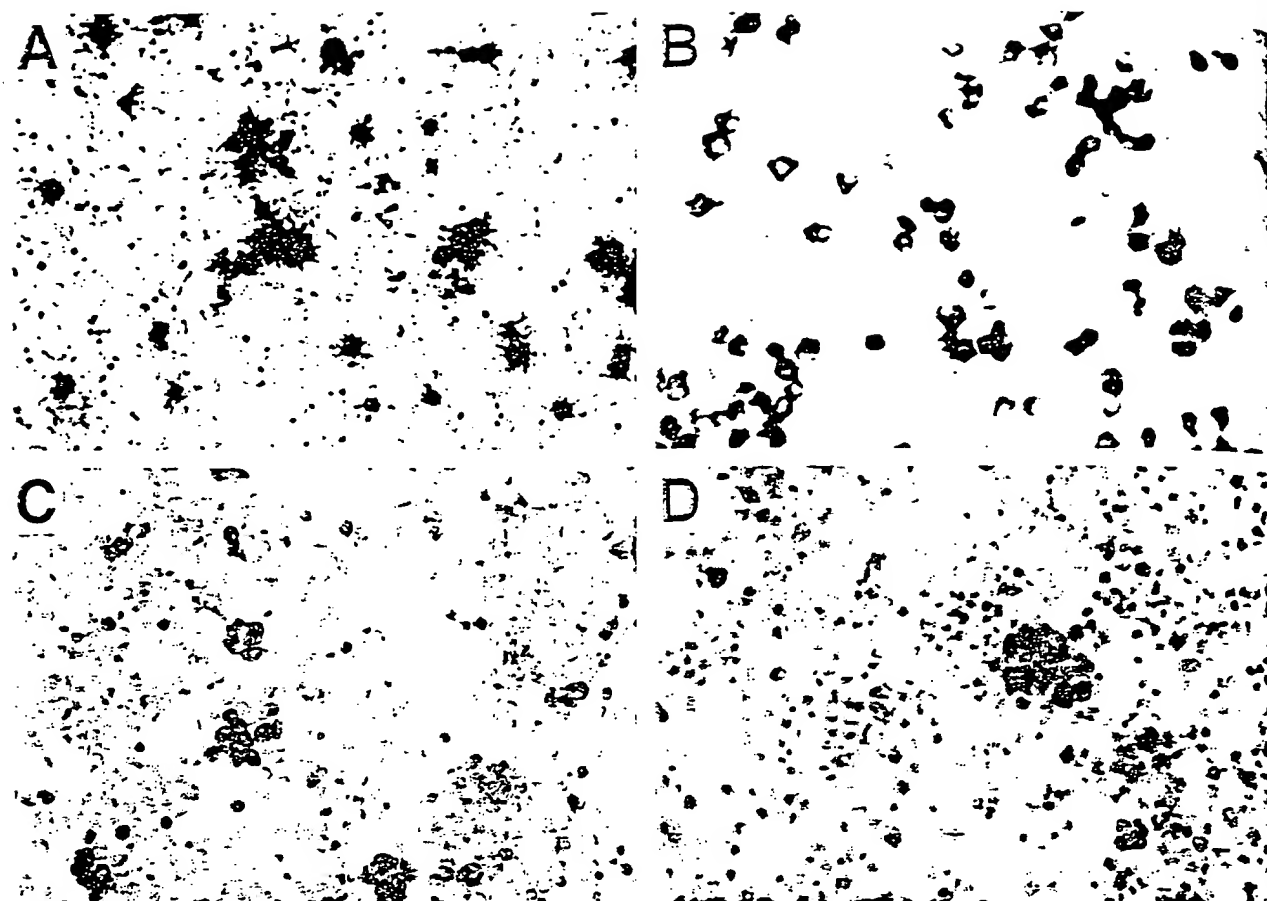


Figure 1. Phenotypic characteristics of ED 14-15 rat FLEC. Fetal liver cells were isolated as described in Materials and Methods, washed, and collected on slides. A: AFP mRNA expression (the fetal form), evaluated by ISH to ^{35}S antisense riboprobe (cells exhibiting autoradiographic grains) in ED 14 FLEC. B: Albumin mRNA expression, evaluated by ISH to digoxigenin-labeled antisense riboprobe (cells exhibiting dark color) in ED 14 FLEC. C: γ -GT, histochemical staining (cells exhibiting dark color) in ED 15 FLEC. D: CK-19, immunohistochemical staining (cells exhibiting dark color) in ED 15 FLEC. Original magnification, $\times 200$.

differentiation. We have analyzed cells isolated from ED 14-15 fetal liver for expression of AFP, albumin, G-6P, γ -GT, CK-19, CK-14, OV-6, and DPPIV. The percentage of FLEC in the total cellular suspension was approximately 15%, determined as the number of cells expressing AFP mRNA (Figure 1A) or albumin mRNA (Figure 1B). None of the isolated ED 14 liver cells expressed G-6P, CK-14, OV-6, or DPPIV (data not shown). However, expression of γ -GT and CK-19 was clearly observed and increased on ED 15 (Figure 1, C and D).

To further characterize the phenotype of immature liver epithelial cells, we tested for coexpression of AFP, albumin, and CK-19 in isolated ED 12 and ED 14 FLEC. The analyses were carried out on cytopspins of isolated cells to allow better evaluation of co-expressed markers in individual cells. Coexpression of AFP and albumin in isolated fetal liver cells was determined by dual label ISH (digoxigenin-labeling of antisense riboprobe specific for the fetal form of AFP mRNA and ^{35}S -labeling of antisense riboprobe for albumin mRNA). The results showed that in ED 12 and ED 14 liver, all cells expressing the fetal form of AFP mRNA coexpressed albumin mRNA (Figure 2, A and B). Coexpression studies using immunohistochemistry for detection of CK-19 and ISH for AFP mRNA showed that most FLEC expressed only AFP mRNA (Figure 2C), some cells expressed both AFP mRNA and

CK-19 (Figure 2C), and a third population of cells expressed only CK-19 (Figure 2D). Dual label immunohistochemistry for CK-19 and ISH for albumin mRNA confirmed this observation, as most FLEC expressed only albumin mRNA, some cells expressed only CK-19, and a third, smaller group of cells coexpressed albumin mRNA and CK-19 (Figure 2: E and F). Dual labeling of isolated 12-day fetal liver cells revealed the same heterogeneity (Figure 2, G and H).

These results demonstrated the existence of at least three phenotypically distinct subpopulations of epithelial cells in 12- to 14-day fetal rat liver. The first group of cells expressed AFP and albumin mRNA but not CK-19. This was the largest subpopulation, comprising roughly 75% of FLEC. The second group (~15% of total) expressed CK-19, but not AFP or albumin mRNA. The third and smallest group of cells (~10% of total) expressed both AFP and albumin mRNAs and CK-19.

Proliferation and Differentiation of FLEC in the Liver of Adult Syngeneic Animals

To follow the proliferation and differentiation of FLEC, we transplanted isolated ED 14 cells into the liver of normal and Rs-treated rats subjected to two-thirds PH. Differen-

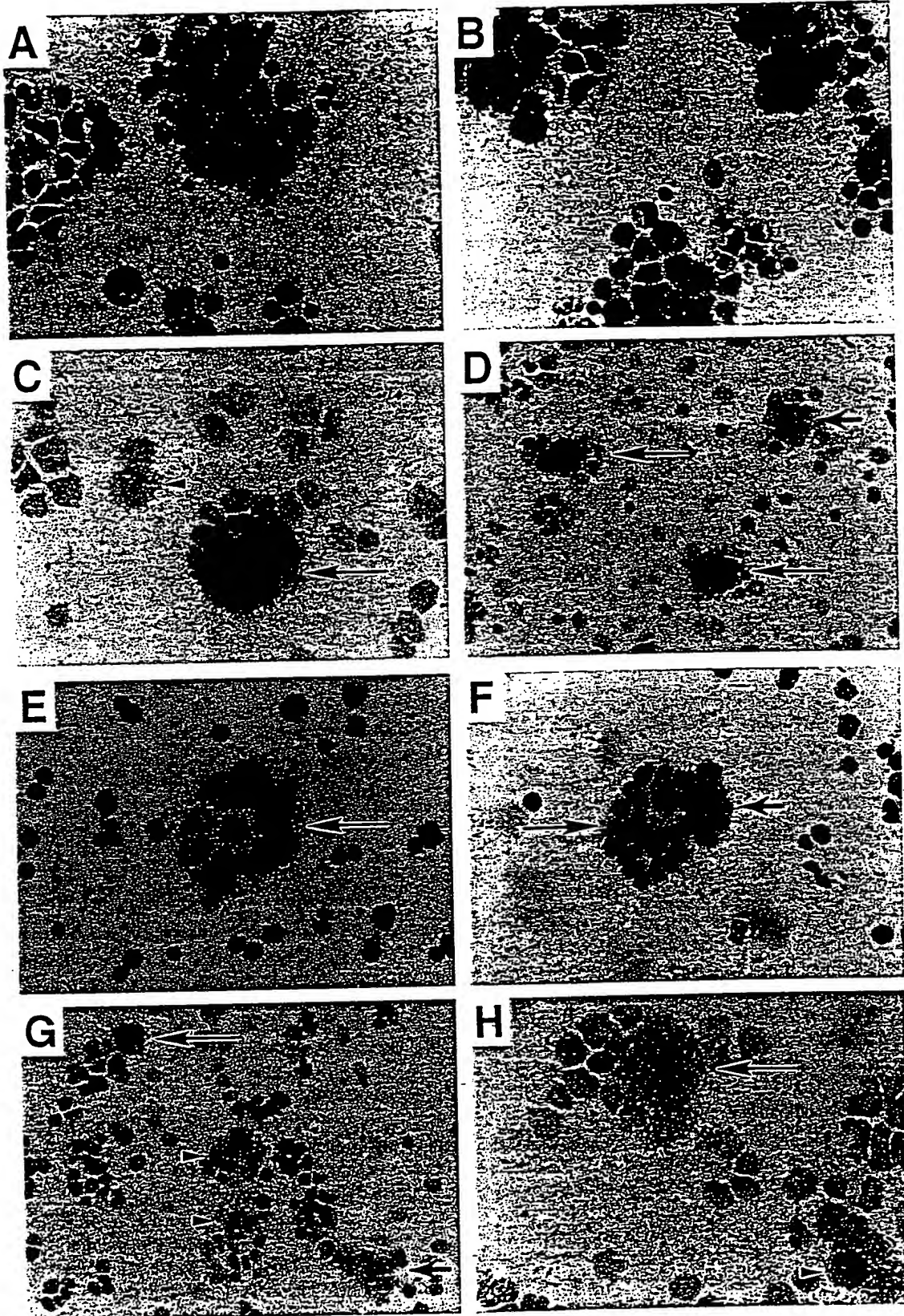


Figure 2. Dual phenotypic characteristics of ED 12 and ED 14 FLEC. Fetal liver cells were isolated as described in Materials and Methods, washed, and collected on slides. Dual ISH for AFP mRNA (brown color) and albumin mRNA (autoradiographic grains) of ED 12 (A) and ED 14 (B) FLEC. All cells expressing AFP mRNA are also positive for albumin mRNA. C and D: Immunohistochemistry (brown color) for CK-19 combined with ISH for AFP mRNA (autoradiographic grains). The majority of cells express only AFP mRNA (arrows in C and D). Some cells expressing AFP mRNA also express CK-19 (arrowhead in C), others express only CK-19 (small arrow in D). E and F: Immunohistochemistry (brown color) for CK-19 combined with ISH for albumin mRNA (autoradiographic grains). Most cells express only CK-19 (small arrow in E), others express only albumin mRNA (arrow in F). G and H: Immunohistochemistry (brown color) for CK-19 combined with ISH for albumin mRNA (autoradiographic grains). Most cells express only albumin mRNA (arrows in G and H). Some cells express only CK-19 (small arrow in G) and others express both albumin mRNA and CK-19 (arrowheads in G and H). Original magnifications: $\times 400$ (A-C, E, F, and H) and $\times 200$ (D and G).

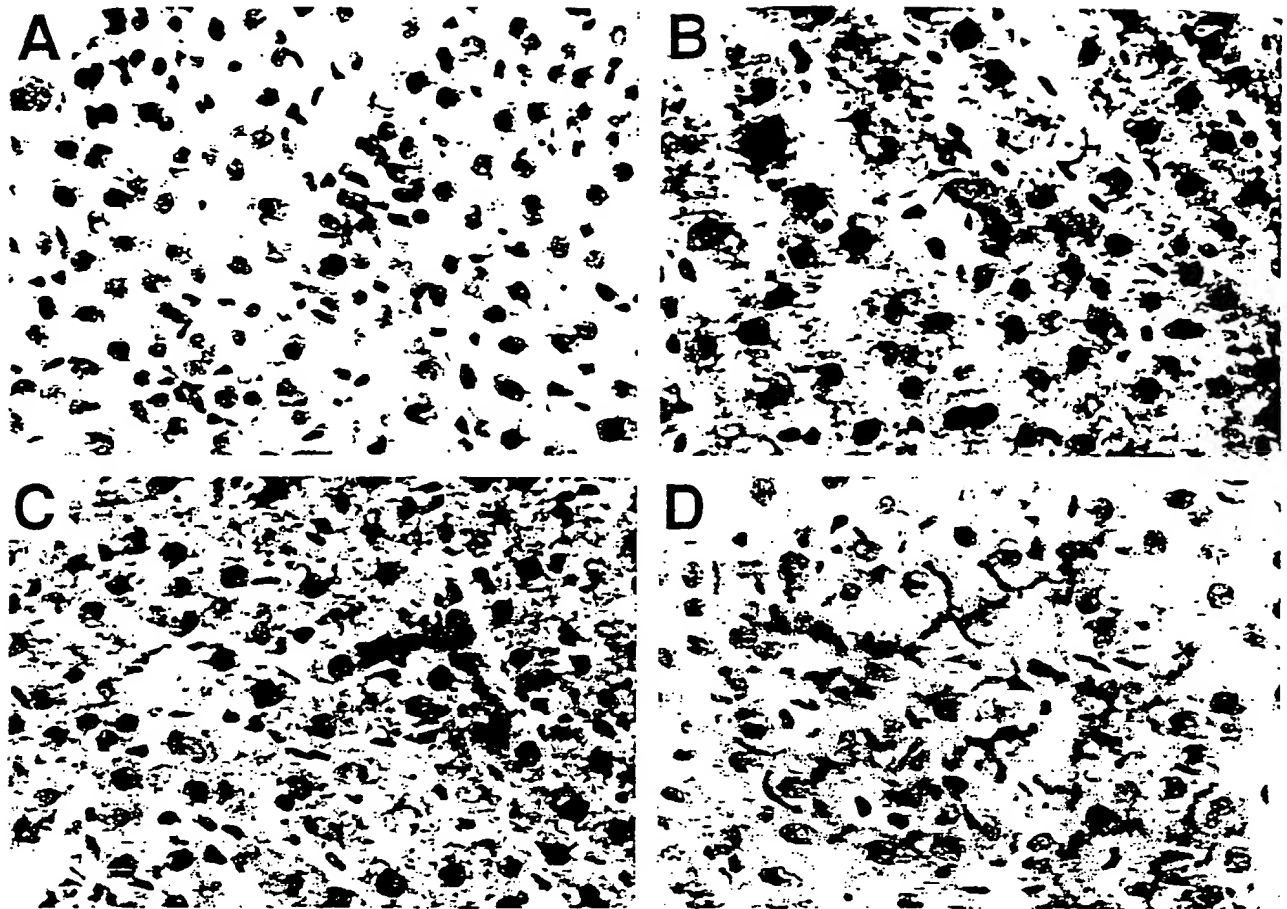


Figure 3. Differentiation of rat FLEC in normal adult regenerating liver. FLEC were isolated from the liver of ED 14 rat DPPIV⁻ fetuses and transplanted into the liver of mutant DPPIV⁻ female rats, as described in Materials and Methods. One, 2 and 4 weeks later, the livers were removed and frozen sections were stained for histochemical detection of DPPIV enzyme activity (red color). **A:** One week after transplantation, scattered cells, diffusely stained for DPPIV, were identified. **B:** Two weeks after transplantation, FLEC in the parenchyma (zones 2 and 3) both expanded in number and differentiated into mature hepatocytes. **C:** Two weeks after transplantation, some cells in the regions of bile ducts (zone 1) had differentiated into BDEC (diffuse staining for DPPIV). **D:** One month after transplantation, larger clusters of morphologically fully differentiated Hc were observed. Fully mature bile duct structures comprised of either transplanted cells or a mixture of transplanted and host cells were also present (data not shown). Original magnification, $\times 400$.

tiation of the cells was monitored morphologically and phenotypically, using DPPIV expression to detect transplanted cells. DPPIV cannot be detected in the liver before ED 16–17 by enzyme histochemistry (our findings), immunoblot,⁴⁹ or indirect immunofluorescence⁵⁰ methods. Therefore, detection of this enzyme served as a marker for both proliferation and lineage progression of transplanted FLEC. In ED 16 liver and thereafter, fetal hepatocytes show diffuse membranous staining for DPPIV. However, in adult liver, hepatocytes show a distinctive and unique expression pattern for DPPIV; it is localized to the apical (bile canalicular) domain of the plasma membrane. On the other hand, mature BDEC still show diffuse membranous staining for DPPIV, so that these two phenotypically distinct liver epithelial cell types can be readily distinguished.

One week after transplantation of FLEC into the liver of normal adult rats, cells scattered throughout the parenchyma were diffusely stained for DPPIV (Figure 3A). This suggested that the cells were not fully differentiated. Two weeks after transplantation, cells in the parenchyma (zones 2 and 3) acquired an hepatocytic morphology with canalicular expression of DPPIV (Figure 3B).

whereas others in the regions of bile ducts (zone 1) differentiated into biliary epithelial cells (Figure 3C). One month after transplantation, larger clusters of DPPIV⁺ cells, on average 2–7 per cm² in random tissue sections, formed morphologically fully differentiated Hc (Figure 3D). In addition, 1 to 2 clusters per cm² exhibited a fully differentiated bile duct epithelial cell morphology (data not shown).

In Rs-treated rats, the kinetics and proportions of various cells repopulating the liver was different from that observed in normal rats. One week after transplantation into Rs-treated animals, many foci or clusters of small epithelial cells with diffuse membranous staining for DPPIV were observed (Figure 4A). Two weeks after transplantation, large foci of DPPIV⁺ hepatocytes were already present in the liver parenchyma (Figure 4B), and once again, DPPIV⁺ bile duct structures were identified in portal regions (Figure 4C). One month after transplantation of FLEC into Rs-treated rats, numerous foci of DPPIV⁺ mature hepatocyte were observed (50–120/cm² in random tissue sections). These foci were beginning to fuse into very large DPPIV⁺ structures, taking over a substantial portion of the liver parenchyma (Figure 4D).

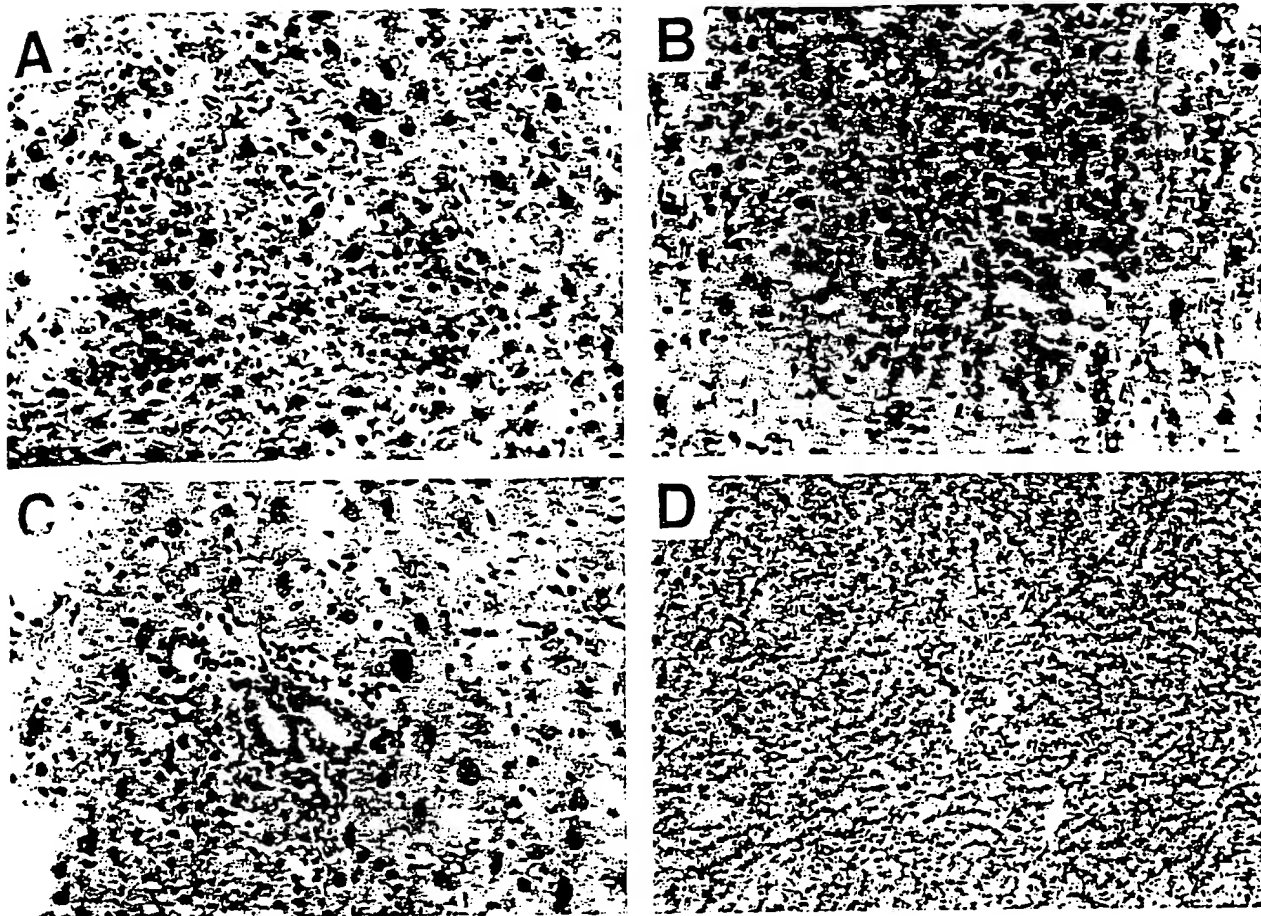


Figure 4. Differentiation of rat FLEC in Rs-treated adult regenerating liver. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted into the liver of mutant DPPIV⁻ female rats treated with Rs, as described in Materials and Methods. One, 2 and 4 weeks later, the livers were removed and frozen sections were stained for histochemical detection of DPPIV enzyme activity (red color). **A:** One week after transplantation, small groups of cells diffusely stained for DPPIV, were found. **B and C:** Two weeks after transplantation, DPPIV⁺ cells formed larger clusters of hepatocytes that were beginning to show canalicular staining for DPPIV (**B**), or BDEC in the bile duct region, as evidenced by diffusely stained DPPIV⁺ small epithelial cells in bile duct-like structures (**C**). **D:** One month after transplantation, numerous clusters of morphologically fully differentiated Hc were observed. Original magnifications, $\times 200$ (**A-C**) and $\times 100$ (**D**).

Although the number of hepatocytic clusters increased considerably in Rs-treated compared to untreated liver after PH and FLEC transplantation, the number of bile duct structures remained unchanged (1–3/cm² in random tissue sections).

To determine whether transplanted cells exhibiting a fully differentiated hepatocyte morphology had lost markers specific for hepatoblasts and acquired markers specific for mature hepatocytes, serial sections from livers taken 1 month after transplantation were processed for DPPIV, CK-19, γ -GT, or AFP mRNA. As shown in Figure 5, DPPIV⁺ hepatocytes did not express CK-19 (Figure 5, **A** and **B**), γ -GT (Figure 5, **C** and **D**), or AFP mRNA (Figure 5, **E** and **F**), although proliferating small epithelial cells in Rs-treated animals express all three of these markers.⁴⁷ The FLEC that differentiated into Hc were functional and expressed very high levels of albumin mRNA (Figure 6, **A** and **B**), and G-6P (Figure 6, **C** and **D**); the latter is not expressed in ED 14 FLEC. Bile duct structures formed by transplanted cells retained expression of CK-19 (not shown) and also became positive for OV-6 (Figure 6, **E** and **F**), another marker that is not expressed in ED 14 FLEC, but is expressed after ED 16 in biliary epithelial lineage-committed cells, as well as in mature BDEC.

These results demonstrate that immature FLEC can proliferate and differentiate into mature Hc and BDEC in regenerating liver of adult syngeneic animals. As noted above, repopulation of Rs-treated liver by transplanted cells occurred much more rapidly than that observed in normal liver. On the other hand, the frequency of appearance of bile duct structures originating from transplanted FLEC was proportionally higher in normal livers.

Proliferation and Differentiation of Immature FLEC into Hc and BDEC Occurs Only in Liver Subjected to a Proliferative Stimulus

In experiments described above, we transplanted 14 day FLEC into normal or Rs-treated adult liver subjected to two-thirds PH. In control experiments, in which PH was not performed, we did not detect DPPIV⁺ cells (Hc or BDEC). In other experiments, animals were subjected to two-thirds PH 1 week after cell transplantation and were then kept for an additional 1, 2, or 4 weeks. All livers (those removed at the time of PH and the regenerated liver 1, 2, or 4 weeks after PH) were analyzed to detect DPPIV⁺ cells. Again, no DPPIV⁺ cells were found either in

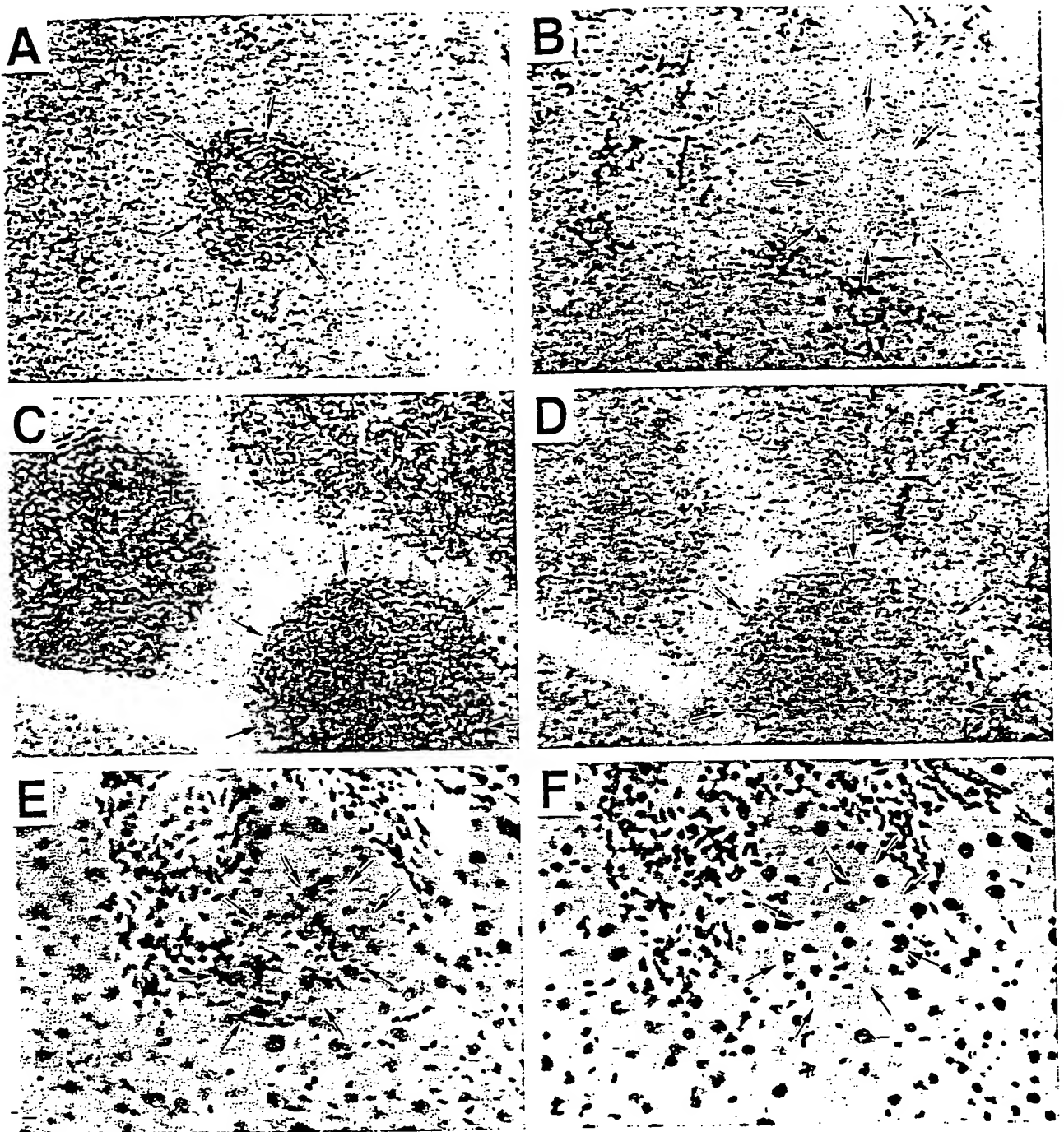


Figure 5. Transplanted cells lose markers of undifferentiated FLEC. Fetal liver cells were isolated from ED 14 rat DPPIV⁺ fetuses and transplanted in conjunction with PH into the liver of mutant DPPIV⁺ female rats treated with Rs, as described in Materials and Methods. Serial sections were processed for DPPIV histochemical staining, shown by transplanted cells exhibiting dark staining in a membranous (bile canalicular) distribution in A, C, and E and for CK-19 by immunohistochemical staining, shown as darkly colored cells in B; γ -GT by histochemical staining, shown as darkly colored cells in D and AFP mRNA by ISH, shown as autoradiographic grains, which are negative, in F. The clusters of transplanted cells are surrounded by arrows. As shown in these serial sections taken 1 month after cell transplantation, DPPIV⁺ hepatocytes are negative for γ -GT, CK-19, and AFP mRNA. Original magnifications, $\times 40$ (A–D) and $\times 200$ (E, F).

the liver removed at the time of PH (1 week after cell transplantation) or in the regenerated liver 1, 2, or 4 weeks after PH. Serial sections from the liver removed 1 week after cell transplantation were also analyzed for clusters of AFP mRNA expressing cells and results were again negative. From these data, we conclude that undifferentiated ED 14 FLEC do not proliferate and differentiate in quiescent liver.

That the proliferative status of the liver is crucial for the proliferation and differentiation of transplanted cells was confirmed by an experiment in which Rs-treated recipient animals received 4 doses of triiodothyronine (T3) instead of PH (see Methods). Recently, we reported that T3 is an alternate mitogen for transplanted adult hepatocytes in Rs-treated liver.⁵¹ One month after transplantation of FLEC, small clusters of DPPIV⁺ mature hepatocytes (Fig-

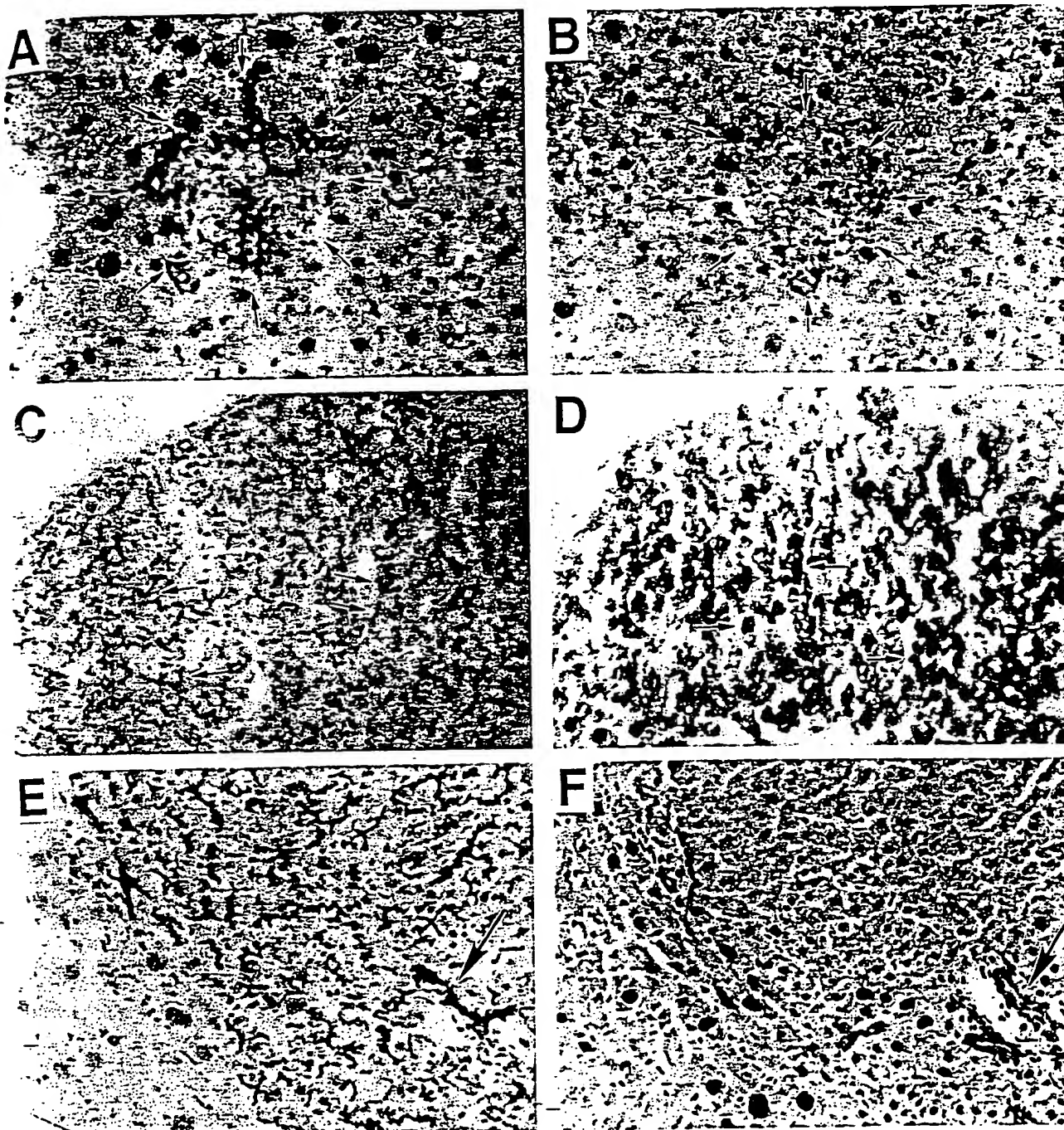


Figure 6. Transplanted cells acquire phenotypic markers of differentiated hepatocytes. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted in conjunction with PH into the liver of mutant DPPIV⁺ female rats treated with Rs, as described in Materials and Methods. One month after cell transplantation, livers were removed and serial sections were processed for DPPIV histochemical staining, (cells with dark staining in a membranous distribution and highlighted by arrows) in A, C, and E. B: ISH for albumin mRNA (autoradiographic grains) in the same region as the DPPIV⁺ Hc in A. This region shows a cluster of transplanted cells with high albumin mRNA expression (circumscribed by arrows). D: Histochemical staining for G-6P (dark color) expressed in Hc originating from transplanted cells in the same large cluster, which fills the microscopic field. F: Immunohistochemical staining for OV-6 (dark color) in epithelial cells within mature bile ducts, some of which are also positive for DPPIV (examples of dual positive cells are highlighted by arrows in E and F). Original magnifications, $\times 200$ (A, B, E, and F) and $\times 100$ (C and D).

ure 7A) or bile duct structures (Figure 7B) were detected in the liver of T3-treated recipients. Although the proliferation of transplanted cells was modest in T3-treated compared to PH treated rats (compare Figures 4D and 7A), this result showed clearly that the recipient liver needs to be activated (subjected to a regenerative stimulus) for

proliferation and differentiation of immature fetal cells to occur.

To determine whether undifferentiated (DPPIV⁺) FLEC remained in the liver, we transplanted FLEC into four normal female rats not subjected to PH and four normal female rats subjected to PH at the time of cell transplan-

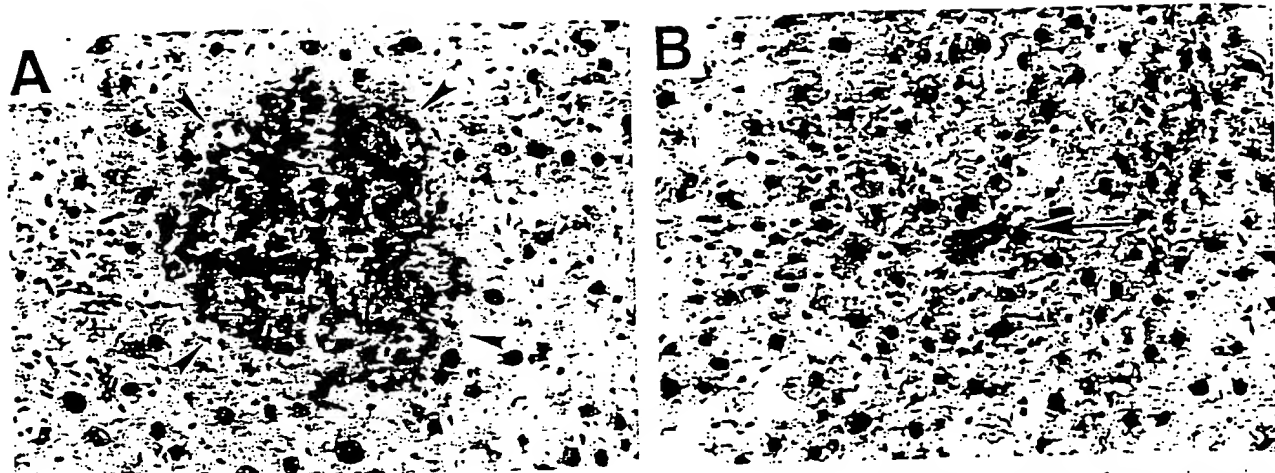


Figure 7. Differentiation of rat FLEC in adult Rs-treated liver stimulated with T₃. FLEC were isolated from the liver of ED 14 rat DPPIV⁺ fetuses and transplanted into the liver of Rs-treated mutant DPPIV⁺ female rats and stimulated with T₃, as described in Materials and Methods. Four weeks later, the livers were removed and frozen sections were stained for histochemical detection of DPPIV. A: Cluster of transplanted cells differentiated morphologically into mature hepatocytes (area surrounded by arrowheads). B: Small bile duct (denoted by large arrow), originating from transplanted cells. Original magnification, $\times 200$.

tation. After 2 weeks, the livers were removed and DNA was isolated from each liver. It was expected that transplanted cells (originating from fetal liver) would be approximately 50% male and 50% female. The presence of male cells was detected by polymerase chain reaction amplification of the *Sry* gene located on the Y chromosome. As shown in Figure 8, some male fetal cells remained in the liver after transplantation in the absence of PH. However, their number was substantially lower than that present in the liver after PH (Figure 8, lanes 1–3 versus lanes 4–6). These cells did not express DPPIV, suggesting that they did not undergo hepatocyte lineage progression in the absence of a liver regenerative stimulus or, alternatively, that they were not of epithelial origin.

Preferential Differentiation of Lineage-Committed FLEC in Normal Adult Liver and of Uncommitted FLEC in Rs-Treated Liver

To determine the proliferative capacity and lineage commitment of the three subpopulations of FLEC described in

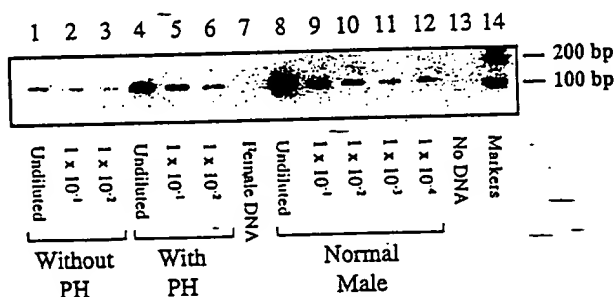


Figure 8. Detection and amplification of the rat *Sry* gene located on the Y chromosome in transplanted cells. DNA was isolated from the liver of female rats 2 weeks after FLEC transplantation. A 104-bp fragment of the *Sry* gene located on the Y chromosome was amplified, as described in Materials and Methods, and resolved on a 2% agarose gel. Lanes 1–3: Amplified fragments from 50, 5, and 0.5 ng, respectively, of recipient DNA, isolated 2 weeks after transplantation. Lanes 4–6: Amplified fragments from 50, 5, and 0.5 ng, respectively, of recipient DNA, isolated 2 weeks after transplantation from animals subjected to PH. Lane 7: Amplified fragment from 50 ng of control female DNA. Lanes 8–12: Amplified fragments from 50, 5, 0.5, 0.05, and 0.005 ng DNA from male F344 rats diluted into control female DNA. Lane 13: control tube with no DNA. Lane 14: Molecular weight markers.

Figure 2, we studied their differentiation in normal and Rs-treated regenerating liver. For this purpose, we analyzed the recipient liver at early time points, ie, 1 and 2 weeks after cell transplantation, following expression and coexpression of AFP mRNA and CK-19 in transplanted cells, which were identified in serial sections by DPPIV enzyme activity.

One week after transplantation of FLEC into normal adult rat liver, scattered DPPIV⁺ cells in the parenchyma were usually AFP mRNA⁺ and CK-19⁺ (Figure 9, A and B). Very infrequently, we found DPPIV⁺ cells that expressed a dual phenotype, ie, they expressed AFP mRNA and CK-19 (Figure 9, C and D). In the periportal region, transplanted DPPIV⁺ cells also expressed CK-19 but did not express AFP mRNA (Figure 9, E and F). The proportion of expanding cells with a single or dual phenotype reflected their relative abundance in the isolated FLEC preparations. Two weeks after transplantation, AFP mRNA expression was still positive, but reduced, in transplanted cells in the parenchyma, and the cells were negative for CK-19 (Figure 9, G and H). DPPIV⁺/CK-19⁺ BDEC did not express AFP mRNA (data not shown).

Different results were obtained in Rs-treated rats. One week after FLEC transplantation, DPPIV⁺ cells formed clusters in the parenchyma expressing AFP mRNA. The larger hepatocytic clusters also expressed CK-19 and thus were bipotential (Figure 10, A and B). However, CK-19 expression was lower in transplanted cells than in endogenous biliary epithelial cells (see arrowhead in Figure 10B). Other DPPIV⁺ clusters (Figure 10C) were comprised of a mixed cell population (CK-19⁺ and CK-19⁻) expressing AFP mRNA (Figure 10D, arrows) and a few clusters of transplanted cells expressed only AFP mRNA (arrowhead). Expression of AFP mRNA was higher in unipotential than in bipotential cells (see Figure 10D). Two weeks after cell transplantation, overall expression of AFP mRNA was reduced, although AFP mRNA was still expressed in both transplanted hepatocytes and biliary epithelium, with the signal being weaker in the latter (Figure 10E and F). AFP mRNA expression in CK-19⁺ hepatocytes in the parenchyma (Figure 10G and H, ar-

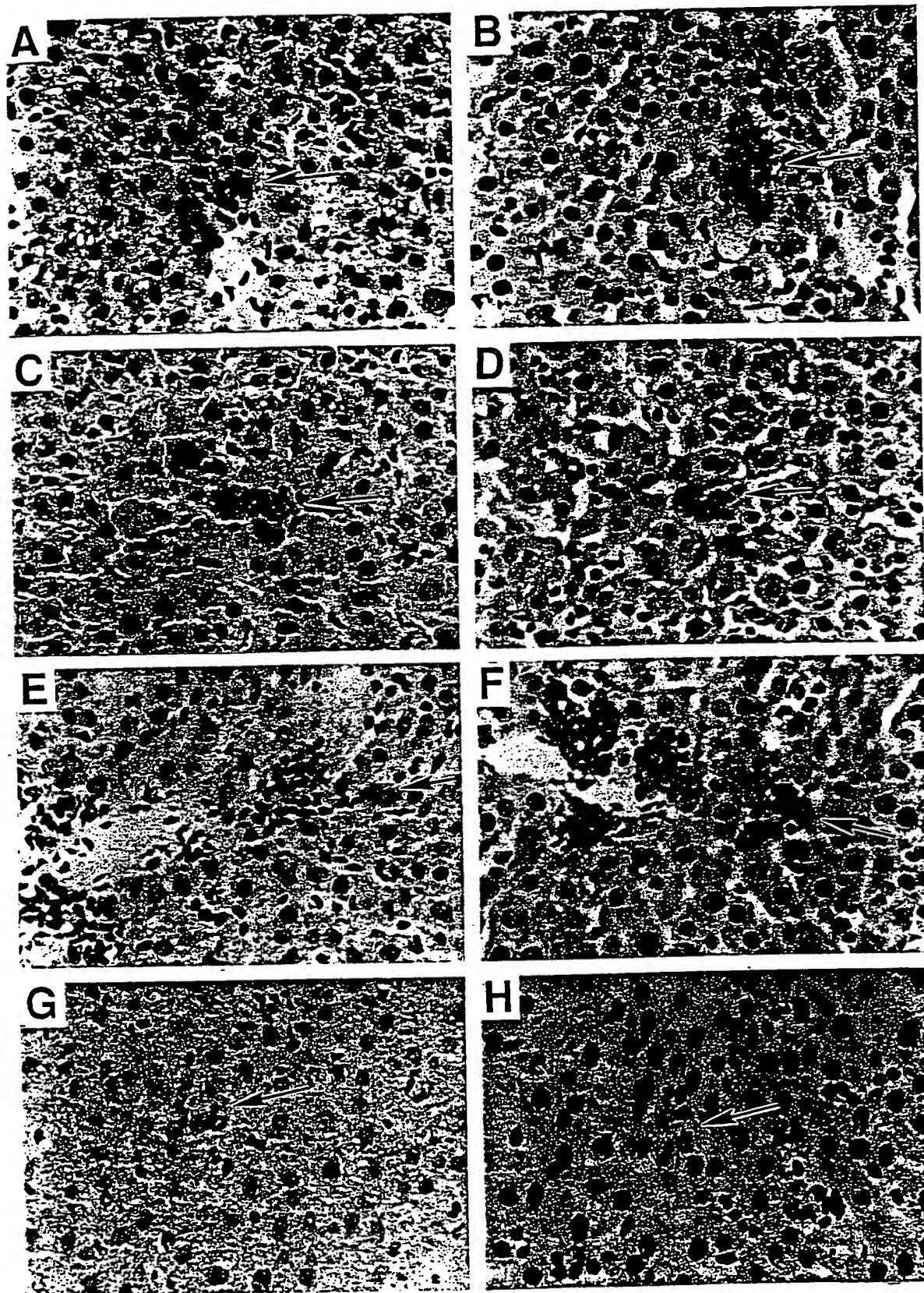


Figure 9. Differentiation of lineage committed FLEC in regenerating liver of normal adult F344 rats. FLEC were isolated from the liver of ED 14 rat DPP-IV^{-/-} fetuses and transplanted in conjunction with PH into the liver of mutant DPP-IV^{-/-} female rats not treated with Rs. One week (A-D) and 2 weeks (E-H) after transplantation, livers were removed and serial sections prepared. Histochemical detection of DPP-IV (red color) is shown in A, C, E, and G and dual immunohistochemical detection of CK-19 (brown color) and ISH for AFP mRNA (autoradiographic grains) is presented in serial sections in B, D, F, and H. B: The cluster of AFP mRNA⁺ cells does not express CK-19. D: AFP mRNA expressing cells do express CK-19. F: None of the CK-19⁺ cells forming bile duct structures (arrow) express AFP mRNA. G and H: Decreased expression of AFP mRNA and absent expression of CK-19 in transplanted cells that differentiated into hepatocytes (arrow). Original magnification, $\times 400$.

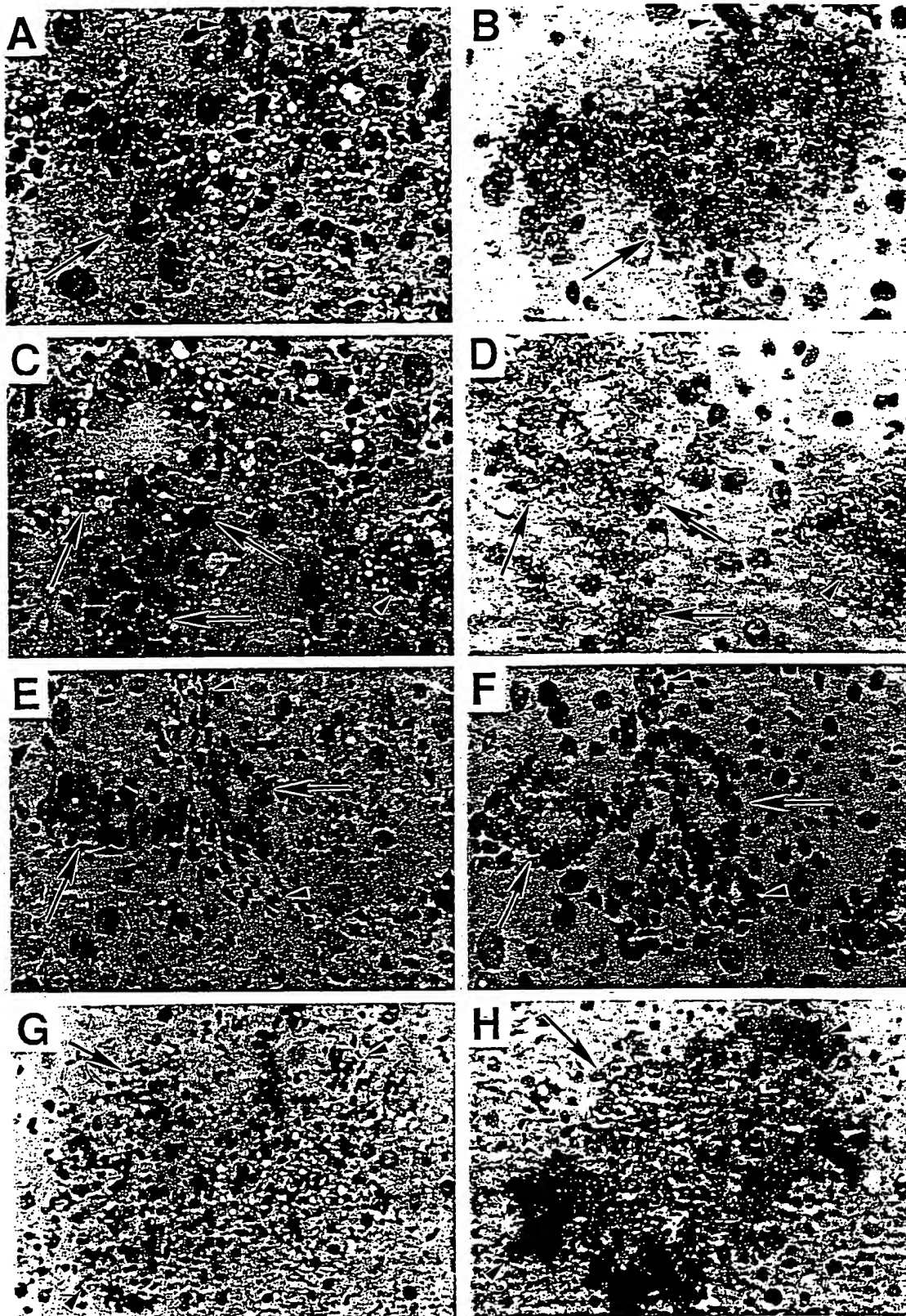


Figure 10. Differentiation of lineage uncommitted FLEC in regenerating liver of Rs-treated adult F344 rats. FLEC were isolated from the liver of ED 14 rat DPPIV^{-/-} fetuses and transplanted in conjunction with PH into the liver of mutant DPPIV^{-/-} female rats treated with Rs. One week (A-D) and 2 weeks (E-H) after transplantation, livers were removed and serial sections processed. Histochemical detection of DPPIV (red color) is shown in A, C, E, and G and dual immunohistochemical detection of CK-19 (brown color) and ISH for AFP mRNA (autoradiographic grains) is shown in B, D, F, and H. B: Transplanted cells, shown in A, formed a large cluster of AFP mRNA⁺ and CK-19⁺ cells, shown in B. The expression of CK-19 is lower than in endogenous small epithelial cells (arrowhead in B). D: A mixed population of transplanted cells, expressing AFP mRNA. Those with lower expression of AFP mRNA also express CK-19 (arrows). The cells with higher expression of AFP mRNA do not express CK-19 (arrowhead). F: Transplanted cells, forming bile duct structures, express both CK-19 and AFP mRNA (arrows). Expression of AFP mRNA is generally lower in CK-19⁺ duct cells than that in Hc. Also note that CK-19⁺ AFP⁻ duct cells of host origin are also present (arrowheads). H: Expression of AFP mRNA in CK-19⁺ Hc (arrow) decreased faster than that in Hc not expressing CK-19 (arrowhead). Original magnifications, $\times 400$ (A-F) and $\times 200$ (G and H).

row) was lower than in hepatocytes expressing only AFP mRNA (Figure 10G and H, arrowheads).

From these studies, it appears that most FLEC that proliferate and differentiate after transplantation into normal liver are of a single phenotype; they express either AFP/albumin or CK-19 and behave like unipotential progenitor cells, differentiating into Hc or BDEC, respectively. In contrast, the bulk of the cells that proliferate and differentiate in Rs-treated liver behave like bipotential progenitor cells; they show a dual phenotype, expressing both AFP and CK-19, and differentiate into either hepatocytes or bile duct epithelial cells. This indicates that the bipotential cells have a higher proliferative capacity than unipotential cells. Because the same cell preparations were used for transplantation into both normal and Rs-treated liver, we conclude that the proliferative status of the recipient liver controls proliferation and lineage progression of the various subpopulations of FLEC.

Discussion

Results reported in this study represent the first demonstration that 14-day immature FLEC can proliferate extensively and differentiate in the liver of adult animals into morphologically and phenotypically mature Hc and BDEC. However, terminal differentiation and expansion of ED 14 FLEC occurs only in a liver subjected to PH or another proliferative stimulus (such as T3 administration). Fetal liver cells cannot complete terminal differentiation in the quiescent-adult liver, normal or Rs-treated. Under these circumstances, most of the transplanted cells are eliminated, although some appear to remain as undifferentiated, dormant stem-like cells that cannot readily be activated. Thus, it can be speculated either that regenerating liver provides the necessary environment, factors, and signals for hepatoblasts to proliferate and differentiate, or that the quiescent liver inhibits these processes. We favor the former hypothesis, as both adult hepatocytes³⁸ and fetal hepatocytes after ED 16 (Sandhu J, Dabeva MD, Petkov PM, Hurston E, Shafritz DA, unpublished results) survive and undergo modest proliferation in Rs-treated liver in the absence of PH.

Preferential Proliferation and Differentiation of Committed FLEC in Normal Liver and Uncommitted FLEC in Rs-Treated Liver

Studying the antigenic profile of ED 12 fetal liver cells, Hixson et al⁵² found three major subpopulations: one expressing only HBD.1, another expressing only OC3, and a third expressing both markers. The authors suggested that all ED 12 cells are transitional and bipotential, and that they originate from a common pre-ED 12 precursor. That hepatic tissue of ED 12 is composed of bipotential epithelial cells that give rise to Hc and BDEC also has been suggested by others studying the differential expression of cytokeratins, AFP, albumin, cell surface markers, and kinetics of appearance of liver-specific markers in the developing rat embryo.^{7-9,29,30} kinetics of

increased expression of one marker and loss of another marker in human embryos,¹⁶ and differential expression of these markers in cell lines under the influence of different promoting agents.^{8,11,13}

From our study, we cannot conclude that there is a specific precursor/product relationship between the different subpopulations of FLEC, as we have observed all three subpopulations from ED 12 up to birth (data not shown). However, our data strongly suggest that commitment toward the hepatocytic or bile duct lineage occurs either very early during formation of the liver diverticulum (before ED 12), or that there is not a single FLEC precursor, as CK-19⁺ cells not expressing AFP may have a separate developmental origin. As demonstrated by cell transplantation, a substantial proportion of ED 14 FLEC are already committed to one or the other lineage, hepatocytic (AFP⁺/albumin⁺) and bile ductular (CK-19⁺). In normal regenerating liver, most of the AFP⁺ transplanted cells, which were scattered throughout the parenchyma, did not express CK-19. CK-19⁺ transplanted cells were found specifically in zone 1 of the liver lobule, as part of bile duct structures. Since on the same sections we observed AFP⁻/CK-19⁺ BDEC and AFP⁺/CK-19⁻ Hc, it is highly unlikely that the differences found in gene expression patterns are due to technical factors or that the cells have lost their dual phenotype 1 week after transplantation. (The expression of AFP in the neonatal liver decreases gradually and shuts off only after 4 weeks of age.)

The third subpopulation of FLEC has a dual phenotype (AFP⁺/albumin⁺ and CK-19⁺) and the cells behave like bipotential progenitors of Hc and BDEC. Our studies provide direct evidence for differentiation of epithelial progenitor cells with dual markers into Hc and BDEC after transplantation into the regenerating liver of Rs-treated animals. These cells exhibit a significantly higher proliferative capacity than endogenous liver cells, taking over approximately 20% of the liver mass within 1 month after cell transplantation and PH.

FLEC as a Source for Liver Cell Transplantation

The results in the present study demonstrate that immature FLEC in the environment of adult regenerating liver can proliferate, differentiate, and express genes characteristic of adult hepatocytes/bile duct epithelial cells. This strongly suggests the potential use of these cells for transplantation and ex vivo gene therapy. A few attempts have been made to transplant ED 18 and older fetal liver cells into the spleen or on solid supports implanted intraperitoneally.⁵³⁻⁵⁶ In all these cases, fetal hepatocytes engraft, proliferate to some extent, and perform liver-specific biochemical functions. Isolated fetal hepatocytes from late gestation, when transplanted intraportally into Nagase albuminemic rats, engraft, expand, and give partial correction of serum albumin when an hepatic regenerative stimulus (portal branch ligation) is also applied.⁵⁷ Several studies also report successful engraftment and differentiation of early fetal liver tissue or cell suspensions after transplantation into ectopic sites.^{7,35,58}

However, engrafted liver tissue masses at ectopic sites do not expand very much, and it is unlikely that such limited liver transplantation will have broad therapeutic application.

The present study suggests that immature FLEC may represent a preferred source of hepatic cells for transplantation compared to adult hepatocytes for the following reasons: 1) FLEC are small (10–12 μm) and their intraportal injection is better tolerated than transplantation of mature hepatocytes (20–35 μm); 2) the number of injected cells we have used for the current experiments is ~5 times lower than the number of adult hepatocytes used for liver repopulation at the same efficiency in our previous study;³⁸ 3) due to their small volume, FLEC are not trapped in the periportal region, where the highest concentration of transplanted adult hepatocytes is observed,⁵⁹ and they move easily through the sinusoids, reaching zone 3 of the liver lobule. This increases the seeding and repopulating efficiency of the transplanted FLEC compared to hepatocytes; 4) immature FLEC possess sufficiently high proliferative capacity that they can repopulate the normal regenerating liver in the absence of Rs treatment; and finally, 5) FLEC differentiate morphologically and phenotypically into both mature hepatocytes and bile duct epithelial cells, which is not observed after hepatocyte transplantation. Since early fetal liver epithelial progenitor cells selectively proliferate in the normal liver in response to a regenerative stimulus (or hepatic parenchymal loss), they differentiate into mature hepatocytes and bile duct epithelial cells, and they become incorporated into the host liver lobule as part of normal hepatocytic cords and bile duct structures, this suggests that fetal liver cell transplantation represents an attractive method to restore functional liver tissue.

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Successful Peripheral T-Lymphocyte-Directed Gene Transfer for a Patient With Severe Combined Immune Deficiency Caused by Adenosine Deaminase Deficiency

By Masafumi Onodera, Tadashi Ariga, Nobuaki Kawamura, Ichiro Kobayashi, Makoto Ohtsu, Masafumi Yamada, Atsushi Tame, Hirofumi Furuta, Motohiko Okano, Shuzo Matsumoto, Hitoshi Kotani, Gerard J. McGarrity, R. Michael Blaese, and Yukio Sakiyama

Ten patients with adenosine deaminase deficiency (ADA⁻) have been enrolled in gene therapy clinical trials since the first patient was treated in September 1990. We describe a Japanese ADA⁻ severe combined immune deficiency (SCID) patient who has received periodic infusions of genetically modified autologous T lymphocytes transduced with the human ADA cDNA containing retroviral vector LASN. The percentage of peripheral blood lymphocytes carrying the transduced ADA gene has remained stable at 10% to 20% during the 12 months since the fourth infusion. ADA enzyme

activity in the patient's circulating T cells, which was only marginally detected before gene transfer, increased to levels comparable to those of a heterozygous carrier individual and was associated with increased T-lymphocyte counts and improvement of the patient's immune function. The results obtained in this trial are in agreement with previously published observations and support the usefulness of T lymphocyte-directed gene transfer in the treatment of ADA⁻SCID.

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ADENOSINE DEAMINASE (EC3.5.4.4; ADA) is an enzyme in the purine salvage pathway that is critical for the deamination of adenosine and deoxyadenosine and consequent formation of inosine and deoxyinosine, respectively. The deficiency of ADA impairs the function of the human immune system resulting in severe combined immunodeficiency (SCID) characterized by severe T lymphocyte dysfunction and agammaglobulinemia.¹⁻³ The clinical course of inherited ADA deficiency (ADA⁻) ranges from the rapidly fatal, early onset of classical ADA⁻SCID to the minimally dysfunctional immune system of patients presenting "partial" ADA deficiency.^{4,5} A recent review classified ADA deficiency into four types as determined by the age at clinical onset and suggested that these variants are the result of different, specific mutations resulting in various severities of enzyme dysfunction.⁶

Although the current treatment of choice for ADA⁻SCID is an HLA-matched bone marrow transplant,⁷ less than one third of patients have access to an appropriate donor. An alternative is enzyme replacement using polyethylene glycol-modified bovine ADA (PEG-ADA). This represents a life saving, but costly, therapeutic option for the patients that do not have an HLA-matched donor.^{8,9} Although enzyme replacement with PEG-ADA partially reconstitutes the immune function of most

patients with ADA⁻SCID, a few patients have been unresponsive to PEG-ADA.

The determination of the complete sequence of both the ADA cDNA¹⁰⁻¹² and the genomic ADA structural gene¹³ has facilitated the molecular analysis of ADA⁻ patients and permitted identification of various genetic mutations in unrelated ADA⁻ patients. Early identification of the mutant gene led ADA⁻SCID to become the first disorder to be treated by gene therapy. Two ADA⁻SCID patients who had manifested differing levels of severity of persistent immunodeficiency despite continuous treatment with PEG-ADA thus were enrolled in 1990.¹⁴ Since then, 10 patients with ADA⁻SCID have undergone gene therapy as recently described.¹⁴⁻¹⁷ The strategies adopted in these trials have differed and the efficacy of treatment has varied.

We report the molecular analysis of the genetic defect in an ADA⁻SCID patient enrolled in the first gene therapy protocol in Japan and analyze the clinical results obtained during the first 18 months of this clinical trial.

MATERIALS AND METHODS

Cell culture. B-lymphoblastoid cell lines (B-LCL) were established from our ADA⁻SCID patient, his parents and a healthy volunteer by Epstein-Bar Virus (EBV) transformation. B-LCL were maintained in RPMI-1640 medium (GIBCO-BRL, Grand Island, NY) with 10% fetal calf serum (FCS; GIBCO-BRL) and 50 mmol/L β-mercaptoethanol (Sigma Chemical Co, St Louis, MO).

Sequence analysis of patient's ADA cDNA and genomic DNA. For the analysis of the ADA cDNA sequence, total cellular RNA was isolated from B-LCL using TRIZOL Reagent (GIBCO-BRL). First-strand cDNA was synthesized from 2 μg of total cellular RNA (First strand synthesis kit; Promega, Madison, WI). Full-length ADA cDNA fragments extending from the translation start site codon to 230 base pair (bp) 3' of the stop codon were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). Oligonucleotide primers for RT-PCR were as follows: sense primer; CCATGGCCCAGACGCCCGCCTT, antisense primer; ACCATAGCCCATGTGCAAGGGC. Reactions containing 0.5 μL (2.5 U) *Taq* polymerase (*TaKaRa Ex Taq*, *TaKaRa Shuzo Co, Ltd*, Tokyo, Japan) were incubated for 30 cycles of 60 seconds at 92°C, 90 seconds at 58°C, and 180 seconds at 72°C with the extension time at 72°C increased to 10 minutes in the last cycle. Amplified products were isolated from 1.0% agarose gel and then subcloned into pCR II vector (Invitrogen, San Diego, CA). Sequence analysis of double-stranded DNA was performed using Sequenase version II DNA sequencing kit (Amersham Life Science, Arlington

From the Department of Pediatrics, Hokkaido University School of Medicine, Sapporo, Japan; Clinical Gene Therapy Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD; Institute of Medical Science, Health Sciences University of Hokkaido, Ainosato, Sapporo, Japan; and Genetic Therapy, Inc, Gaithersburg, MD.

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Address reprint requests to Yukio Sakiyama, MD, PhD, Department of Pediatrics, Hokkaido University School of Medicine, North 15, West 7, Kita-ku, Sapporo, 060 Japan.

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Heights, IL) with [35 S]dATP (Amersham Life Science) and a series of ADA-specific primers. Amplified products were sequenced through a 6% acrylamide gel (National Diagnostics, Atlanta, GA). To analyze the ADA genomic sequence, high molecular DNA was obtained from B-LCL by standard techniques.¹⁸ Primers and PCR conditions for amplification of ADA all exons have also been described previously.¹⁹⁻²¹ Amplified products were isolated from agarose gel and sequenced directly using the Thermal Cycler DNA sequencing kit (Circum Vent; New England Biolabs Inc, Beverly, MA). ADA cDNA sequences are numbered relative to the start site of translation and genomic DNA according to Wiginton et al.¹³

Southern blot analysis. High molecular weight DNA from B-LCL was digested with restriction endonuclease *Rsa* I, separated in 1.0% agarose gel, and transferred onto a nylon membrane (Biotrace HP; Gelman Sciences, Ann Arbor, MI). Filters were then hybridized to a 32 P randomly labeled 444-bp *Rsa* I-*Pst* I fragment from the ADA cDNA.

Retroviral-mediated gene transfer into patient's peripheral T cells. The clinical protocol used here has been described elsewhere.²² Briefly, peripheral T lymphocytes from the patient were obtained by apheresis (CS3000 plus, Baxter Corp, Chicago, IL), isolated by density gradient centrifugation, and then maintained in AIM-V medium (GIBCO-BRL) supplemented with 5% FCS (GIBCO-BRL), 100 U/mL of recombinant human IL-2 (rIL-2, SHIONOGI, Osaka, Japan) and 10 ng/mL of anti-CD3 antibody (Orthoclone OKT3 Injection; Ortho, Raritan, NJ) in gas-permeable culture bags (Nipro Pretobag; Nishyo, Osaka, Japan). After 72 hours, half of the medium was removed and replaced with supernatant containing the LASN retroviral vector²³ supplemented with interleukin-2 (IL-2) and 10 μ g/mL of protamine (Shimizu, Shimizu City, Japan). The LASN supernatant, prepared under Good Manufacturing Practices guidelines, was supplied by Genetic Therapy Inc (Gaithersburg, MD). The transduction procedure was repeated twice following an optimized transduction protocol combining low-temperature (32°C) incubation and centrifugation.²⁴ After two rounds of transduction, the virus supernatant was replaced with fresh medium supplemented with IL-2 and the cells were cultured for an additional 6 days. At the 11th day of culture, the cells were harvested and washed extensively with saline containing 0.5% human albumin and then reinfused into the patient.

Analysis of the inserted proviral genome by semi-quantitative PCR. Sense (GAGGCTGTGAAGAGCGGCAT) and anti-sense (CTC-GAAGTGCATGTTTCCT) primers were designed to match the sequence of the start site of exon 7 and the end of exon 8, respectively. Using these primers, the amplification of DNA samples from vector-containing cells generates two bands; the larger one (250 bp) derived from the endogenous ADA gene containing intron 7 (76 bp) and the smaller one (174 bp) from the LASN provirus. To evaluate the frequency of transduced cells in the patient's peripheral blood, a standard curve was prepared from a serial dilution of in vitro-transduced and G418-selected B-LCL with untransduced cells. The ratio of the amount of amplified ADA cDNA derived from the integrated vector and the amplified genomic sequence was calculated after hybridization with an ADA cDNA probe.

Thin-layer chromatography (TLC) analysis of ADA enzyme activity. Mononuclear cells were washed twice with phosphate-buffered saline to remove FCS and then suspended in 100 mmol/L Tris, pH 7.4 containing 1% bovine serum albumin. Cell lysates were obtained by 5 rapid freeze-thaw cycles. Cellular debris was removed by centrifugation and the lysates were stored at -80°C until used. ADA enzyme activity was assayed by the measurement of the conversion of [14 C] adenosine (Amersham Life Science) to [14 C] inosine and [14 C] hypoxanthine followed by TLC separation of the reaction products performed as previously described.²⁵ The results were expressed as nanomoles of inosine and hypoxanthine produced per min by 10^6 cells (nmol/min/ 10^6 cells).

RESULTS

Clinical course. The patient is a 5-year-old Japanese male. Symptoms including a chronic productive cough and a purulent nasal discharge began at 8 months of age. At 10 months he developed respiratory distress and was hospitalized for the treatment of severe pneumonia that was unresponsive to antibiotics. On admission at age 10 months, the patient had lymphopenia (absolute lymphocyte count 520/ μ L), with few mature T and B lymphocytes (CD3, 125/ μ L; CD4, 62/ μ L; CD8, 41/ μ L; CD19, 26/ μ L) and low serum Ig levels (IgG, 342 mg/dL; IgA, 18 mg/dL; and IgM, 60 mg/dL). Both humoral and cellular immunity were defective, with undetectable isohemagglutinins and absent T-cell proliferative responses to phytohemagglutinin, Concanavalin A, and pokeweed mitogen. Since ADA activity in his red blood cells (RBCs) was undetectable and the deoxyadenosine triphosphate (dATP) level was 506 nmol/mL RBCs (normal <2 nmol/mL), the diagnosis of SCID due to ADA deficiency of the "delayed onset" type⁶ was established. In the absence of a suitable bone marrow donor, PEG-ADA therapy was initiated at 15 months of age and supplemented with intravenous Ig (IVIG). After treatment with PEG-ADA (37.5 U/kg/wk), the plasma ADA activity in the patient's peripheral blood increased from 0.14 to 53.15 μ mol/h/mL and the peripheral blood lymphocyte (PBL) count increased to the range of 1,000 to 2,000/ μ L. Despite continuous PEG-ADA treatment, however, his Ig levels remained below normal and the lymphopenia recurred during the second year of enzyme replacement. The PBL count decreased to less than 1,000/ μ L with CD3⁺ cell counts of 400/ μ L before the start of gene therapy (PBL, 702/ μ L; CD3, 400/ μ L; CD4, 205/ μ L; CD8, 191/ μ L; CD19, 57/ μ L on protocol day 0).

Identification of mutations responsible for ADA deficiency. To analyze mutations in our patient, we amplified full-length ADA cDNA from the patient's EBV transformed B-LCL by RT-PCR. Sequence analysis revealed that all of the clones (6/6) carried a G⁶³² to A transition resulting in replacement of the arginine residue by histidine at codon 211 (Fig 1A). The mutation eliminates a recognition site for the restriction enzyme *Rsa* I. We took advantage of this feature to distinguish the mutated allele from the normal allele.¹⁹ High molecular weight DNA extracted from the patient's B-LCL was digested with *Rsa* I, blotted and hybridized to an ADA cDNA probe spanning the region from this mutation site in exon 7 to the end of exon 11 (Fig 1B). *Rsa* I digestion showed both a normal (3.1 kb) and a larger fragment (4.4 kb) in the patient lane, indicating that the patient was heterozygous for loss of the *Rsa* I recognition site in exon 7. To determine the parental derivation, amplified genomic fragments spanning intron 6 to intron 9 of the patient and his parents were digested with *Rsa* I and electrophoresed in 2% agarose gel (Fig 1C). The patient's digestion pattern was identical to that obtained from the analysis of the father's DNA, indicating that this mutation was derived from the paternal allele.

Northern blot analyses showed that the quantity of the ADA message from both the patient and his mother was reduced to approximately half of control (data not shown). All cDNA clones carried the paternal missense mutation, suggesting that the mutation derived from the maternal allele resulted in undetectable mRNA. To characterize this mutation, we ana-

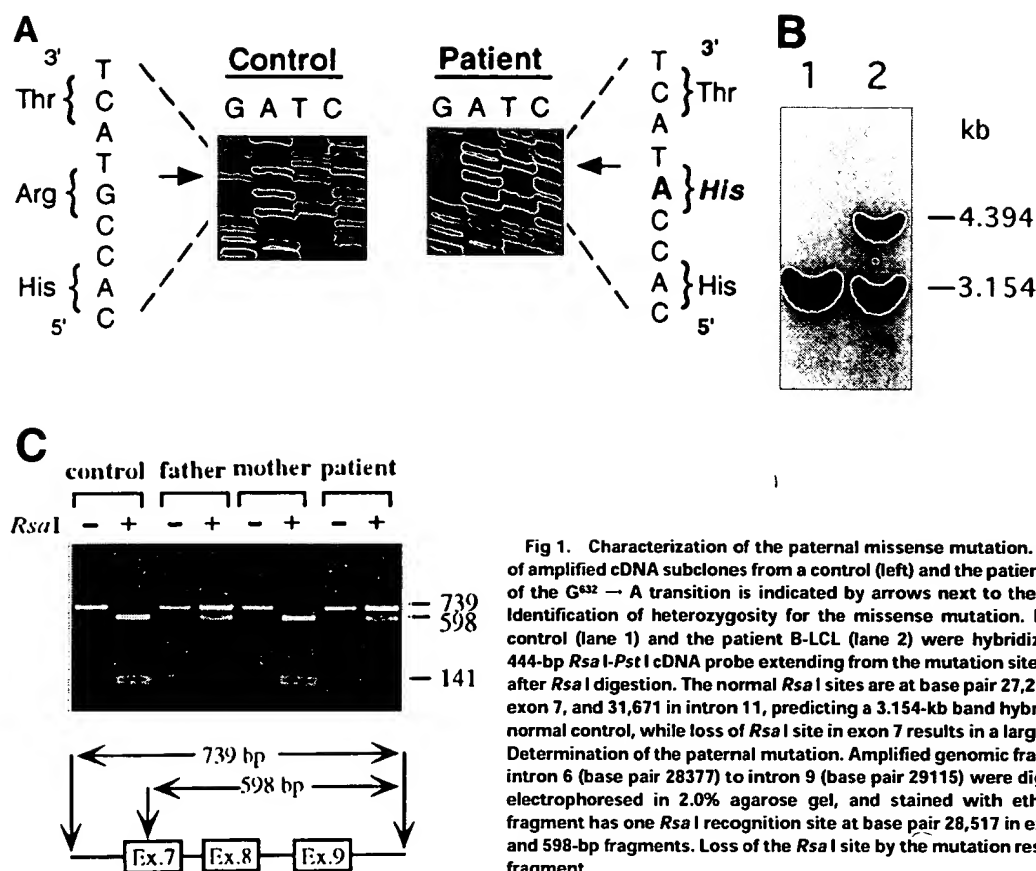


Fig 1. Characterization of the paternal missense mutation. (A) Sequence (sense) of amplified cDNA subclones from a control (left) and the patient (right). The position of the $G^{632} \rightarrow A$ transition is indicated by arrows next to the sequence ladder. (B) Identification of heterozygosity for the missense mutation. DNA samples from a control (lane 1) and the patient B-LCL (lane 2) were hybridized to a radiolabeled 444-bp *Rsa*I-*Pst*I cDNA probe extending from the mutation site to the end of exon 11 after *Rsa*I digestion. The normal *Rsa*I sites are at base pair 27,276 in exon 6, 28,516 in exon 7, and 31,671 in intron 11, predicting a 3.154-kb band hybridized to the probe in normal control, while loss of *Rsa*I site in exon 7 results in a larger band (4.394 kb). (C) Determination of the paternal mutation. Amplified genomic fragments (739 bp) from intron 6 (base pair 28377) to intron 9 (base pair 29115) were digested with *Rsa*I and electrophoresed in 2.0% agarose gel, and stained with ethidium bromide. The fragment has one *Rsa*I recognition site at base pair 28,517 in exon 7, predicting 141- and 598-bp fragments. Loss of the *Rsa*I site by the mutation results in an undigested fragment.

lyzed exons 1 to 11 by PCR amplification of genomic DNA and direct sequencing. Sequence analyses of the amplified fragments including exon 2 showed the patient to be heteroallelic for a splice site mutation at the first position of intron 2 ($G^{+1} \rightarrow A$ transversion) (Fig 2A). This mutation eliminates a recognition site for the restriction enzyme *Bsp*MI. *Bsp*MI digestion showed that the patient and his mother were heterozygous for this mutation, while the father showed a normal individual digestion pattern (Fig 2B). Reports of mutation analyses of other patients have shown that a mutation affecting a mRNA splicing mechanism may give rise to a nonfunctional or unstable mRNA.^{26,27} This mechanism is also supported by the fact that *Rsa*I digestion showed that all full-length cDNA clones (48/48) from the patient's B-LCL carried the paternal G^{632} to A missense mutation.

Retroviral mediated gene transfer into peripheral T cells. At the age of 4, the patient was enrolled in a clinical gene therapy trial that repeated the protocol of the first gene therapy experiment at the National Institutes of Health (NIH) in 1990.²² The patient's peripheral mononuclear cells, obtained by apheresis, were stimulated with IL-2 (100 U/mL) and anti-CD3 antibody (OKT3; 10 ng/mL). After 72 hours of stimulation, they were transduced twice during the next 48 hours by exposure to the ADA retroviral vector LASN, expanded 20- to 50-fold in number by culturing for 6 days after the beginning of transduction, and then reinfused into the patient (see Materials and Methods). No selection procedure to enrich for gene-transduced

cells was performed. Semiquantitative PCR of the cells in the first and second infusions revealed that the frequency of the vector-carrying cells ranged from 3% to 7% (data not shown).

Clinical course after gene therapy. The patient received a total of 10 infusions over the 18-month period (Fig 3). A striking increase in lymphocyte number was observed early in the trial, followed by a gradual return to the basal level. This was followed by a sustained increase after the 8th infusion (protocol day 322) and the patient's PBL count has since remained in the normal range (PBL, 1,980/ μ L; CD3, 1,822/ μ L; CD4, 240/ μ L; CD8, 1,538/ μ L; CD19, 154/ μ L on protocol day 429). Progressive inversion of CD4/CD8 ratio has been observed since the 4th infusion due to an increase of the absolute CD8⁺ cell count. This phenomenon is thought to be the result of preferential proliferation of CD8⁺ cells during in vitro culture and transduction. ADA enzyme activity, nearly undetectable in the patient's lymphocytes before gene therapy, also increased progressively after the 7th infusion (protocol day 252) and reached 27 U on protocol day 476, which is approximately comparable to that of a heterozygous carrier individual (the patient's mother, 34.8 U).

The number of transduced cells in the patient's peripheral blood were assessed by semiquantitative PCR using PBL obtained before each infusion (Fig 4). The frequency of the genetically modified cells increased with the number of infusions of the ADA gene transduced lymphocytes and exceeded 10% of total circulating mononuclear cells just before the 5th infusion (on protocol day 126; Fig 4, lane 4). The frequency

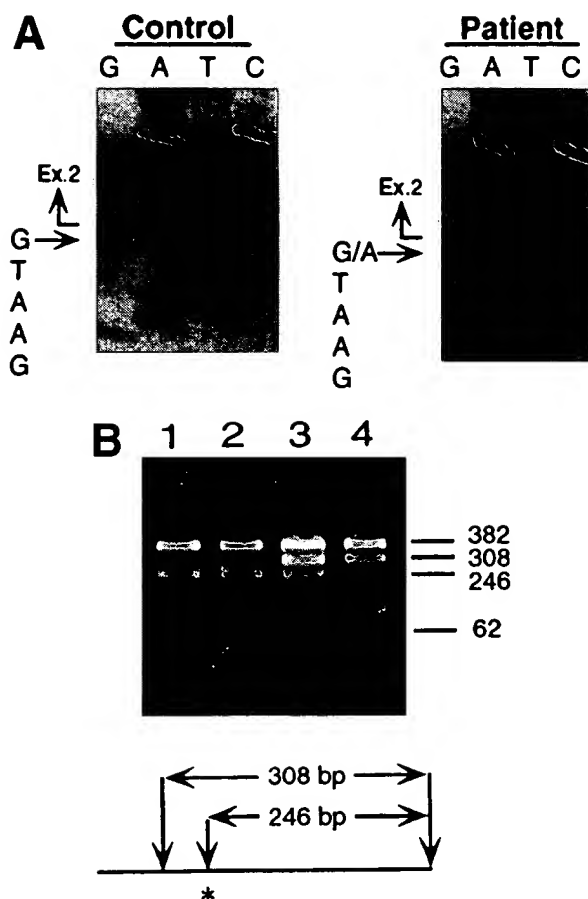


Fig 2. Identification of the maternal mutation at the splice donor site in intron 2. (A) Sequence (sense) of the exon 2/intron 2 junction in amplified genomic DNA. Genomic fragments containing exon 2 were amplified from a control (left) and the patient (right) and sequenced directly. A mutation at the splice donor site in intron 2 ($G \rightarrow A$) is indicated by arrows. (B) Detection of the splice site mutation by the *Bsp*MI digestion. Amplified genomic fragments (690 bp) from intron 1 (bp 14,901) to intron 2 (base pair 15,590) was digested with *Bsp*MI, electrophoresed in 2.0% agarose gel, and stained with ethidium bromide. The fragment has two *Bsp*MI recognition sites at bp 15,282 and 15,344, predicting 62-, 246-, and 382-bp fragments in the control lane. Loss of the *Bsp*MI site (base pair 15,282) by the mutation results in the undigested fragment (308 bp). Lane 1, control; lane 2, father; lane 3, mother; and lane 4, patient. *Bsp*MI digestion shows the patient and his mother were heterozygous for the splice site mutation.

measured before each of the 6th through 10th infusions (on protocol days 210 to 462) has remained stable at 10% to 20%.

To evaluate the functional consequences of the ADA enzyme activity that had been induced by gene transfer, we compared the patient's immune function before and after the treatment (Table 1). Eleven months after beginning gene therapy, the patient's isohemagglutinin titer (IgG) increased from undetectable to 1:16 and delayed-type hypersensitivity (DTH) skin test responses became stronger. The interval between IVIG infusions which were given monthly before gene therapy, was widened and eventually stopped after gene therapy. Despite this, the patient's serum Ig levels gradually increased and have

remained normal for more than a half year without additional IVIG treatment (Fig 3 and Table 1). These results suggest that the accumulated genetically corrected T lymphocytes in the patient's peripheral blood are associated with improvement of cellular and humoral immune responses and an increase in his circulating lymphocyte count. Although he sometimes became transiently febrile after infusions, the patient showed no serious adverse reactions to the treatments.

DISCUSSION

Advances in molecular biology during the past 3 decades have suggested that gene transfer could provide a new approach to the treatment of inherited diseases as well as acquired disorders such as cancer and acquired immune deficiency syndrome.²⁸ The number of active gene therapy protocols has increased greatly since the first clinical gene therapy trial.²⁹ ADA-SCID is one of the few early candidate disorders suitable for such interventions.³⁰ Accordingly, 10 ADA-SCID patients have been enrolled in gene therapy clinical protocols that employed different strategies, retroviral vector designs, and target cell populations. The results obtained from these trials have recently been reported.¹⁴⁻¹⁷

This trial of gene therapy for an ADA-SCID patient in Japan began in August 1995. Over the next 18 months he received a total of 10 infusions of cultured-expanded autologous T cells that had been transduced with the LASN retroviral vector. After an initial period of fluctuating counts, the patient's T cells stabilized in the normal range and this has been sustained for the last half year. The frequency of integrated provirus in the patient's peripheral blood increased to approximately 15% (0.1 to 0.2 proviral copies/cell) by the 4th infusion and has remained stable since that time. The patient's cell associated adenosine deaminase enzyme activity has increased from barely detectable before treatment to values approaching those found in the peripheral mononuclear cells of his heterozygous carrier mother. Delayed hypersensitivity skin tests, a measure of T-cell function, have improved. Isohemagglutinin titers have also increased and his dependence on infusions of normal gammaglobulin has eased. The patient has gained 3 kg in weight during this trial. He is still receiving periodic PEG-ADA replacement and is attending public school with no more infections than his classmates.

The period of observation has simply not been sufficient to assess the full breadth or the duration of this improved clinical status and immune responsiveness. Further, additional studies will be required to reconcile the apparent dissociation between the level of T-cell ADA observed and the proportion of cells containing integrated vector at different time points. Also, the effect of withdrawal of the exogenous PEG-ADA treatment must await more complete characterization of the quality of the patient's immune system and the repertoire of specificities represented in the transduced T-cell population.

Four gene therapy clinical trials including 10 ADA-SCID patients have been performed since the first trial in 1990. Although these trials provided much data that suggested how future gene therapy might be improved by changing retroviral vector design, transduction methods and target cell populations, we found it difficult to compare the efficacy of these various trials because of differences inherent within these basic strate-

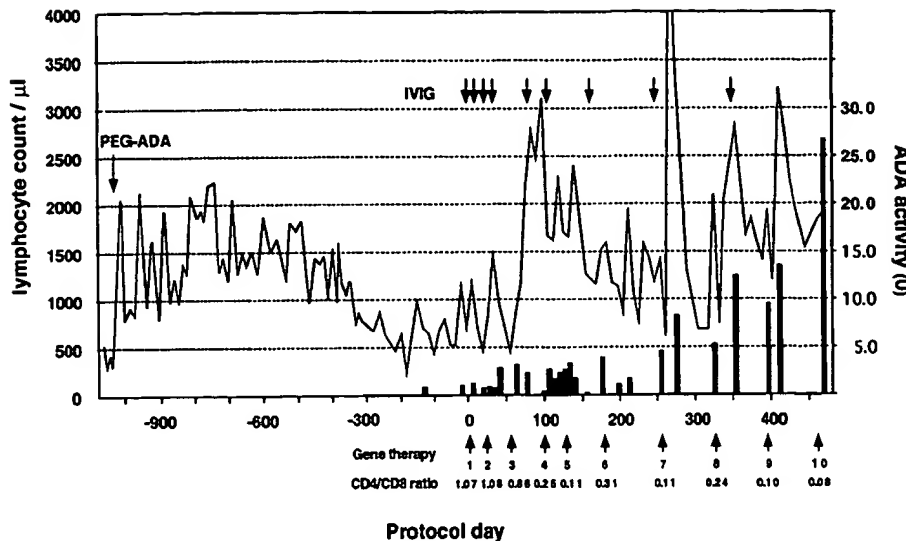


Fig 3. Clinical course before and after gene therapy. Gene therapy started on August 1, 1995 (protocol day 0) with the patient receiving a total of 10 infusions to date. PEG-ADA therapy was initiated at 15 months of age. The lymphocyte count is indicated by a solid line and CD4/CD8 ratio was measured using PBL before infusion. ADA activity shown by a solid bar is expressed as nanomoles of inosine and hypoxanthine produced per minute by 10^6 cells. Replacement of IVIG after gene therapy is shown as an arrow. The patient received a Ig replacement (2.5 g) monthly before gene therapy.

gies. Our trial has been performed following the identical protocol and vector preparations and autologous T lymphocyte isolation procedures that were used in the NIH trial. From this perspective, our trial provides an additional opportunity to evaluate the effectiveness of peripheral T lymphocyte-directed gene therapy for ADA-SCID patients. Interestingly, the clinical course of our patient is quite similar to that observed in patient 1 in the NIH trial. Both trials have shown high gene transfer efficiency, remarkable increase of the ADA enzyme activity and eventual improvement of immune function. In contrast, patient 2 in the NIH trial experienced a low gene transfer efficiency and no significant increase in the ADA enzyme activity even though she exhibited some increase in immunological function. Although the factors leading to this difference have not yet been completely identified, a striking difference in the transduction efficiency of peripheral T cells between the three patients may be relevant. Transduction efficiencies before infusion were 3% to 7% for the present case, 1% to 10% for patient 1 and 0.1% to 1% for patient 2 in the NIH trial. An abbreviated proliferative capacity of patient 2 in the NIH trial was also observed. In addition, a contribution of the development of an immune response to the neomycin resistance gene must be considered since the existence of dominant selectable markers of nonhuman origin may result in unwanted immune reactivity that could eliminate or functionally impair transgene-expressing cells.³¹

The severity of the underlying ADA gene defects could also affect gene transfer. In addition to the mutation analysis reported here, specific ADA gene defects have also been reported for the two NIH patients.²⁰ These three cases can be classified by the severity of their clinical presentation. Both the present case and patient 1 in the NIH trial are of the "delayed onset" type, have splice site mutation defects and have achieved significant levels of "gene-corrected" circulating cells. However, the NIH patient 2 carries compound missense mutations and has manifested low transduction efficiency despite her less severe "late onset" type of presentation at age 5. Although there are insufficient numbers of treated patients to draw firm conclusions at this point, it does appear thus far that the responses of patients with "more severe" gene defects and clinical presentations are at least as responsive as cases with "milder ADA defects."

It should be noted that the ADA gene transduced T lymphocytes possess a selective advantage over the nontransduced cells due to the latter's high intracellular concentration of deoxyadenosine.^{32,33} In the ADA- newborn trial using gene-corrected CD34⁺ cells obtained from the patient's umbilical cord blood,¹⁶ LASN vector was detected in the peripheral blood T cells of these patients at a stable frequency of approximately 0.01% during the first 18 months of observation. Then, after a 50% reduction in their weekly dose of PEG-ADA, the proportion of

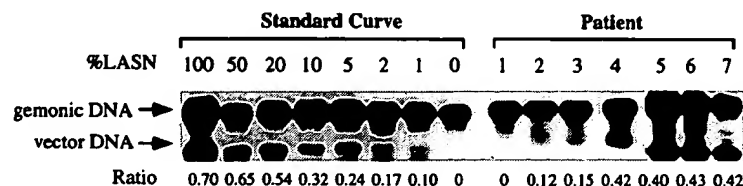


Fig 4. Semiquantitative PCR analysis to evaluate the frequency of vector-carrying cells in the patient's peripheral blood. Patient's mononuclear cells were obtained before the indicated infusion: before gene therapy (lane 1), 2nd infusion (protocol day [D] 21-lane 2), 4th infusion (D 98-lane 3), 5th infusion (D 126-lane 4), 6th infusion (D 175-lane 5), 8th infusion (D 322-lane 6, and 10th infusion (D 462-lane 7), and assayed for the frequency of vector containing cells by semiquantitative PCR. A standard was prepared by diluting cells containing the LASN vector with nontransduced cells. The ratio was determined by comparing the density of the cDNA derived band to that of the genomic DNA derived band.

Table 1. Isohemagglutinin Titer, DTH Skin Test Reactivity, and Ig Levels Before and After Gene Therapy

		Before	After
Isohemagglutinin titer	(IgG)	<2	16
DTH skin test (mm)	PPD*	8 × 7	12 × 10
	Candida	3 × 3	18 × 9
	Tetanus	N.D.	6 × 5
Igs	IgG	720	811
	IgA	20	53
	IgM	84	128

Isohemagglutinin titer and DTH skin tests were tested using standard protocols before gene therapy (before) and at 11 months after the beginning of gene therapy (after) while on PEG-ADA. The patient serum Ig levels were measured just before the Ig replacement on protocol day -60 (before) and 478 (after). The patient received the last Ig replacement at protocol day 348.

*The patient had been immunized with BCG at 5 months of age.

ADA vector-containing T cells in the blood increased to approximately 10% in each case (D.B. Kohn, personal communication, September 1995). In the present case, the dosage schedule of PEG-ADA enzyme has remained constant since the beginning of the trial (18 U/kg/wk on the protocol day 431), during which time the patient's immune function has substantially improved. It might be expected that the proportion of the transduced cells in the patient's PBL will increase as the PEG-ADA dosage is decreased.

To date, three clinical trials have been performed to assess the possibility of treating ADA-SCID patients by correcting hematopoietic progenitor cells.¹⁵⁻¹⁷ The results obtained from these trials suggest that cord blood provides a stem cell population more suitable for efficient retroviral-mediated gene transfer than does bone marrow. Taken with the observations made in the NIH trial, our results strongly suggest that the effectiveness of T lymphocyte-directed gene transfer is a viable addition to the treatment programs that should be considered for ADA-SCID patients. After additional courses of treatment and continued observation to determine the breadth and durability of these positive responses, we hope to reduce or eliminate exogenous ADA enzyme supplementation in this patient. Improvements in vector design to permit higher levels of ADA expression and innovative strategies that provide greater efficiency of stem cell gene transduction may make gene therapy the treatment of choice for ADA-SCID patients.

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*Original Contribution***DEMONSTRATION OF DIFFERENTIATION IN
HEPATOCTE PROGENITOR CELLS USING DIPEPTIDYL
PEPTIDASE IV DEFICIENT MUTANT RATS**SAMUEL H. SIGAL,[‡] PANKAJ RAJVANSHI, LOLA M. REID,[†] and SANJEEV GUPTA*Departments of Medicine, [†]Molecular Pharmacology, [‡]Microbiology and Immunology, and Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York, USA

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Abstract—The presence of progenitor or stem cells in the adult liver and their potential roles in oncogenesis are unresolved issues. The study of hepatocyte progenitor cells has been limited by a lack of convenient in vivo systems allowing unequivocal cell localization and demonstration of differentiation into hepatocytes. To develop an in vivo progenitor bioassay, early (E14) fetal Fischer 344 rat hepatoblasts were transplanted into the spleen of syngeneic, weanling rats deficient in dipeptidyl peptidase IV (DPPIV) activity. The donor status of transplanted hepatoblasts was demonstrated by DPPIV expression. Localization of hepatoblasts was facilitated by the use of an ectopic site, as well as weanling recipients, which readily allowed identification of very small numbers of transplanted cells. Fetal rat hepatoblasts were demonstrated to undergo cellular differentiation along the hepatocyte lineage by acquiring glucose-6-phosphatase activity within 5 d of transplantation. A critical review of previous transplantation studies of hepatocyte progenitor cells and the role of the local microenvironment at inducing differentiation indicates that this novel bioassay should facilitate analysis of progenitor cells.

Keywords—Liver, Progenitor cell, Bioassay, DPPIV, Gene expression

INTRODUCTION

The question of a role for putative hepatocyte progenitor or stem cells in oncogenesis and liver regeneration has elicited much interest. An early event in hepatic oncogenesis is the proliferation of small, periportal cells with oval nuclei, scant cytoplasm and features of fetal hepatoblasts, "oval cells." Based on immunophenotyping, cytology, isozyme profiles, cellular gene expression, and in vivo labeling with ³H-thymidine of sequential histologic sections, activated oval cells are thought to differentiate into hepatocytes or advance to hepatic tumors (Dunsford et al., 1989; Evarts et al.,

1987, 1989; Farber, 1984; Faris and Hixson, 1989; Germain et al., 1988b; Hayner et al., 1984; Hixson et al., 1990; Lemire et al., 1991; Marceau et al., 1989; Sell and Dunsford, 1989; Sell, 1990; Sigal et al., 1992). In contrast, it was recently concluded that oval cells are entirely of the biliary lineage (Tarsetti et al., 1993). Nonetheless, the approaches utilized in previous studies have been indirect as the fate of individual cells was not unequivocally established.

An alternative approach for analyzing cell lineage involves studies with isolated cells. Analysis of liver cells in vitro has been useful, but the precise conditions to induce lineage-specific differentiation remain to be determined (Germain et al., 1988a; Sirica et al., 1990; Thorgeirsson, 1993). This limitation is potentially overcome with a bioassay if differentiating conditions could be reestablished for the transplanted cells. Although differentiation of a nonparenchymal liver epithelial cell line expressing a retrovirally introduced marker gene into hepatocytes has been demonstrated in the liver, induction of differentiation along the hepa-

*Address all correspondence to S. Gupta, M.D., Liver Research Center, Albert Einstein College of Medicine, Ullman 625, 1300 Morris Park Avenue, Bronx, NY 10461, USA.

[†]Department of Physiology, Campus Box #7038, Glaxo Building, Room 34, University of North Carolina School of Medicine, Chapel Hill, NC 27514, USA.

[‡]Alcohol Research Center and Department of Medicine, Bronx Veterans Administration Medical Center, 1300 W. Kingsbridge Road, Mount Sinai School of Medicine, Bronx, NY 10468, USA.

toocyte lineage in ectopic sites has been problematic (Coleman et al., 1993). Thus, the ideal bioassay for hepatocyte progenitor cells—which involves minimal cell perturbation, unequivocal identification *in vivo*, and an ability to analyze small cell numbers is lacking. The advantage of using an ectopic site would be to analyze cellular changes without confusion with host hepatocytes in the liver.

Using a syngeneic substrain of Fischer 344 rats deficient in cell surface dipeptidyl peptidase IV (DPPIV) activity, we developed a novel system to characterize transplanted DPPIV⁺ hepatocyte progenitor cells. Fetal rat hepatoblasts, embryonic age day 14 (E14), were transplanted into the spleen of DPPIV⁻ weanling rats. Prior to E15, fetal rat hepatoblasts are generally uncommitted with respect to hepatocyte and biliary lineages (Germain et al., 1988a; Shiojiri et al., 1991). In the spleen, DPPIV⁺ E14 hepatoblasts engrafted and acquired glucose-6-phosphatase (G-6-P) activity, indicating differentiation along the hepatocyte pathway (Leskes et al., 1971). Because the spleen is an ectopic site, appearance of differentiated function in transplanted hepatoblasts was readily visualized. Furthermore, use of weanling recipients permitted localization of small numbers of transplanted cells. This system should facilitate systematic analysis of various hepatocyte lineages, as well as the role of transforming events and specific agents during carcinogenesis.

MATERIALS AND METHODS

Animals

Female Fischer 344 rats with timed pregnancy and adult male Fischer 344 rats (180–200 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) and maintained on a standard diet with 12 h light cycles. By convention, the first day of gestation was defined as day 0. Weanling DPPIV⁻ rats were bred and provided by the Special Animals Core of the Liver Research Center at the Albert Einstein College of Medicine. Animals were used in accordance with NIH Policy (NIH Publication #86-23, revised 1985), and their usage was approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine.

Fetal liver disruption

Fetal livers on the 14th day of gestation (E14) were disrupted as previously described (Sigal et al., 1994). Briefly, fetal livers were excised, placed in Hank's balanced salt solution containing 0.8 mM MgCl₂, 20 mM HEPES (modHBSS), and gently agitated at room temperature for 1 min. After removal of nonhepatic tissue, livers were triturated and stirred at 37°C for 10

to 15 min in an Erlenmeyer flask with 0.6% Collagenase D (Lot EJA145, Boehringer Mannheim, Indianapolis, IN) in modHBSS containing 0.06% DNase I (Boehringer Mannheim) plus 1 mM CaCl₂. At 5 min intervals, undigested tissue fragments were sedimented at 1 × g, the supernatant recovered, and the process repeated with fresh collagenase solution. The dispersed cells were pooled, suspended in modHBSS containing 0.5 mM EGTA and filtered through a 46 µm tissue collector (Bellico Glass, Inc., Vineland, NY) under 1 × g. The resultant cell suspension was centrifuged at 4°C for 5 min under 400 × g, washed with modHBSS containing 0.1% bovine serum albumin (BSA), and then resuspended in RPMI 1640 culture medium prior to transplantation.

Isolation of adult rat hepatocytes

To perfuse the liver, a solution containing 10 mM HEPES, 3 mM KCl, 130 mM NaCl, 0.5 mM NaH₂PO₄·7H₂O, and 10 mM D-glucose was adjusted to pH 7.4. Animals were sedated with pentobarbitone, and the portal vein cannulated. The solution was warmed to 37°C and infused at 20 ml/min with aeration through a silastic tubing. The initial 5-min of perfusion were with buffer containing 1.9 g EGTA/L, 1 min with buffer that did not contain EGTA, and the final 15 to 20 min with buffer containing 0.025% (w/v) collagenase D (Boehringer Mannheim) plus 280 mg CaCl₂·2H₂O/L (Sigma Chemical Co., St. Louis, MO). After dissociation, cells were filtered through an 80 micron dacron mesh and washed twice at 50 × g for 1 min each. Cell viability was tested by trypan blue exclusion.

AFP and albumin immunocytochemistry

Cytospun cells were fixed with ice-cold ethanol or alcohol, acetone and carbowax 1540 (Fix-Rite; Richard-Allan Medical Industries, Richland, MI). Antibody binding was detected with the biotin/streptavidin method using a beta-galactosidase system (BioGenex, San Ramon, CA). Primary antibodies included rabbit anti-mouse AFP antiserum (ICN ImmunoBiologicals, Costa Mesa, CA) and rabbit anti-rat albumin IgG (U.S. Biochemical Corp., Cleveland, OH). Negative controls consisted of cells incubated without the primary antibody.

Hepatoblast transplantation

DPPIV⁺ donor hepatoblasts were from normal Fischer 344 rats. Recipients were DPPIV⁻ syngeneic Fischer 344 rats, in which DPPIV is totally inactive

(Thompson et al., 1991). Under ether anesthesia, weanling recipient rats weighing 40–50 g were subjected to a left subcostal laparotomy and the spleen exteriorized. A ligature was placed to encircle the lower pole of the spleen for hemostasis at the end of the procedure. Using a 26G needle, cells from three to four fetal livers containing approximately 0.5×10^6 hepatoblasts which had been suspended in 0.1 ml medium were injected directly into the splenic pulp. The abdominal incision was then closed in layers and animals observed until recovery from anesthesia. At selected intervals, animals were killed, and excised spleen and liver frozen to -70°C in OCT resin (Miles Inc., Elkhart, IN) and stored for further studies.

DPPIV staining

Cryostat tissue sections were fixed in chloroform and acetone (1:1, v/v) for 10 min at 4°C . After air-drying, sections were incubated for 30 to 45 min at room temperature in a solution containing 0.4 mg Glycyl-L-proline-4-methoxy-2-naphthylamide substrate with 1.0 mg of Fast Blue B salt in phosphate buffered saline, pH 7.4, at room temperature (Sigma Chemical Co.), and the reaction discontinued by washing with water. Sections were lightly counterstained with methyl green and mounted in glycerol.

G-6-P staining

Unfixed cryostat sections were stained as per Teutsch (1978). Tissue sections were first rinsed with 300 mM sucrose and immersed in the substrate solution for 10 min at room temperature. The substrate solution was prepared by combining 0.2 ml of 200 mM Tris Maleate (pH 6.5), 0.3 ml of 1 M sucrose, 0.1 ml of 100 mM G-6-P (disodium salt), 0.05 ml of 2% lead nitrate and 1.0 ml water. Sections were rinsed in 300 mM sucrose and then dipped in a dilute solution of ammonium sulfide in water for up to 15 s until optimum development of color. The reaction was stopped by washing slides with water. Unless noted, sections were counterstained with methyl green and mounted in glycerol.

RESULTS

Characterization of fetal liver cells

The protocol used for disrupting fetal liver provided single cells in suspension. The viability of isolated cells ranged from 90 to 100% and was in agreement with our previous findings (Sigal et al., 1994). At E14 there are approximately 2×10^6 cells/liver. A large fraction of cells are of hemopoietic, especially erythro-

poietic, origin as indicated by their morphology. Flow cytometric (FACS) analysis of E14 liver cells using a combination of specific endothelial and myeloid antibodies showed that E14 cells were in many respects comparable to E15 liver cells (Sigal et al., 1994; data not shown). AFP⁺ or albumin⁺ hepatoblasts constituted only a small proportion of the total (6 to 8%). Cytologically, hepatoblasts defined by AFP or albumin positivity were characterized by variable amounts of vacuolated cytoplasm (Fig. 1). None of the E14 cells were positive for G-6-P, which is in agreement with the previous findings that G-6-P is only expressed after E18 (Leskes et al., 1971). Selection of liver cells by immunoadsorption with rat red blood cells (RBC) and nonparenchymal cell antibodies significantly depletes contaminating hemopoietic and endothelial cells. Despite further cell selection by FACS, such enriched cell populations retain heterogeneity as previously described (Sigal et al., 1994). Since the goal of this study was to develop a bioassay which permitted analysis of differentiation in early hepatoblasts along the hepatocyte lineage, utilization of progenitor cells immediately after hepatic dissociation and removal of embryonic stroma was adequate.

Cell differentiation and fate in vivo

Transplanted adult hepatocytes maintain cellular gene expression in liver and spleen, as well as peritoneal cavity (Gupta et al., 1994). Although the hepatic microenvironment supports cell engraftment, survival and differentiation (Coleman et al., 1993; Gupta et al., 1991), there is significant dilution in numbers of visible transplanted hepatocytes after dispersal throughout the hepatic parenchyma (Ponder et al., 1991). To develop a bioassay applicable to relatively restricted numbers of progenitor liver cells, we focused on the potential of the weanling spleen for in vivo analysis. When the spleen was recovered from recipients (approximately 1 to 2×10^5 hepatoblasts/animal), cell engraftment was readily apparent (Fig. 2). There were numerous epithelial cells in the spleen with a morphology similar to adult hepatocytes. Transplanted cells were unequivocally localized by their DPPIV positivity, exhibited abundant cytoplasm, and were scattered singly or in groups within the splenic red pulp. Transplanted cells were identified from recipients at various intervals, up to the 8 weeks duration of the experiment.

In adult hepatocytes, DPPIV activity is largely detected in the bile canaliculus, which is a specialized hepatocellular organelle (Arias et al., 1993; Fukui et al., 1990). Localized areas of intense staining for DPPIV activity in transplanted fetal liver cells indicate a morphological response similar to adult hepatocytes

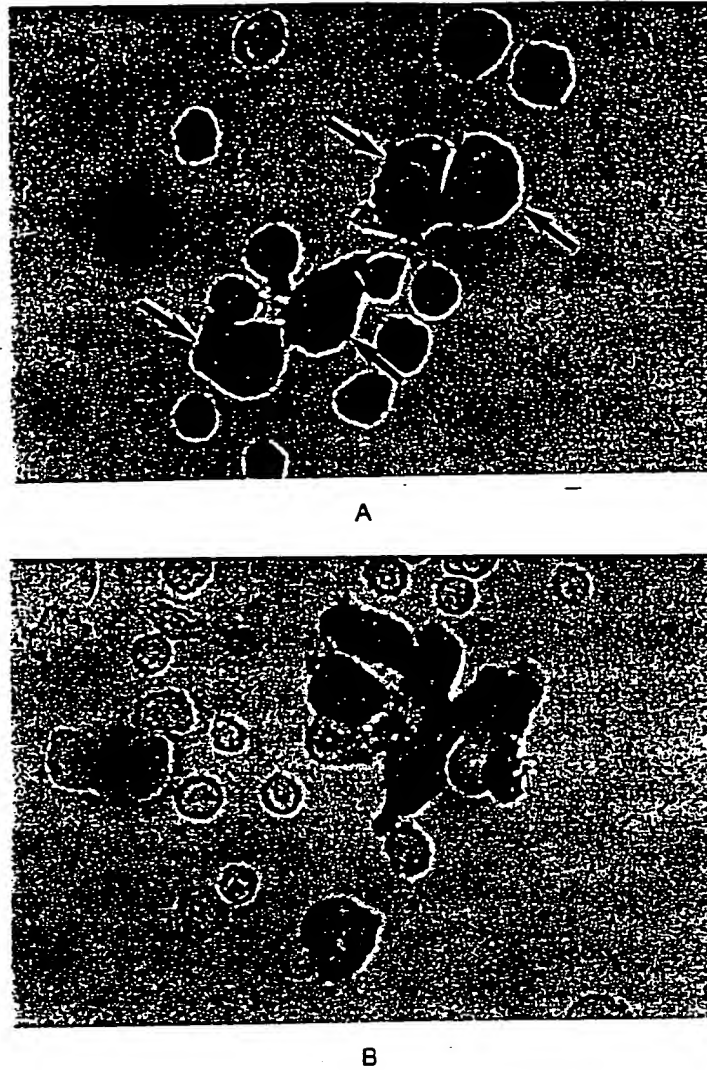


Fig. 1. Albumin and AFP immunostaining in isolated fetal progenitor cells. (A) AFP positive cells were faintly stained and appeared to be larger with vacuolated cytoplasm (arrows), compared with AFP negative cells. (B) A cluster of albumin positive fetal progenitor cells. These cells were also larger with vacuolated cytoplasm. Use of antibodies to immunostain these antigens is described in methods.

in the spleen. In addition, G-6-P activity was apparent in transplanted fetal hepatoblasts when examined as early as 5 d posttransplant, consistent with maturation along the hepatocyte lineage.

After injection into spleen, cells translocate into hepatic sinusoids (Gupta et al., 1991; Ponder et al., 1991). To study whether fetal liver cells survived in hepatic sinusoids, we analyzed DPPIV activity in tissues from recipients. Cells with DPPIV activity were present in the liver from recipients at day 5, as well as at week 8 of transplantation (Fig. 3). Hepatoblasts were observed to enter host liver cell plates, although DPPIV activity remained somewhat diffused compared with bile canalicular DPPIV activity in adult liver. Some transplanted cells also persisted in hepatic sinusoids,

which is consistent with the relatively heterogeneous cell population used, as littoral cells may have been present.

DISCUSSION

Our most significant findings reported here include development of a novel system for studying maturation of undifferentiated progenitor liver cells along the hepatocyte lineage, utility of the splenic microenvironment to support cell engraftment and differentiation, and analysis of even a few cells using the weanling recipients.

In developing a hepatocyte progenitor cell bioassay, it is essential that the microenvironment used permits

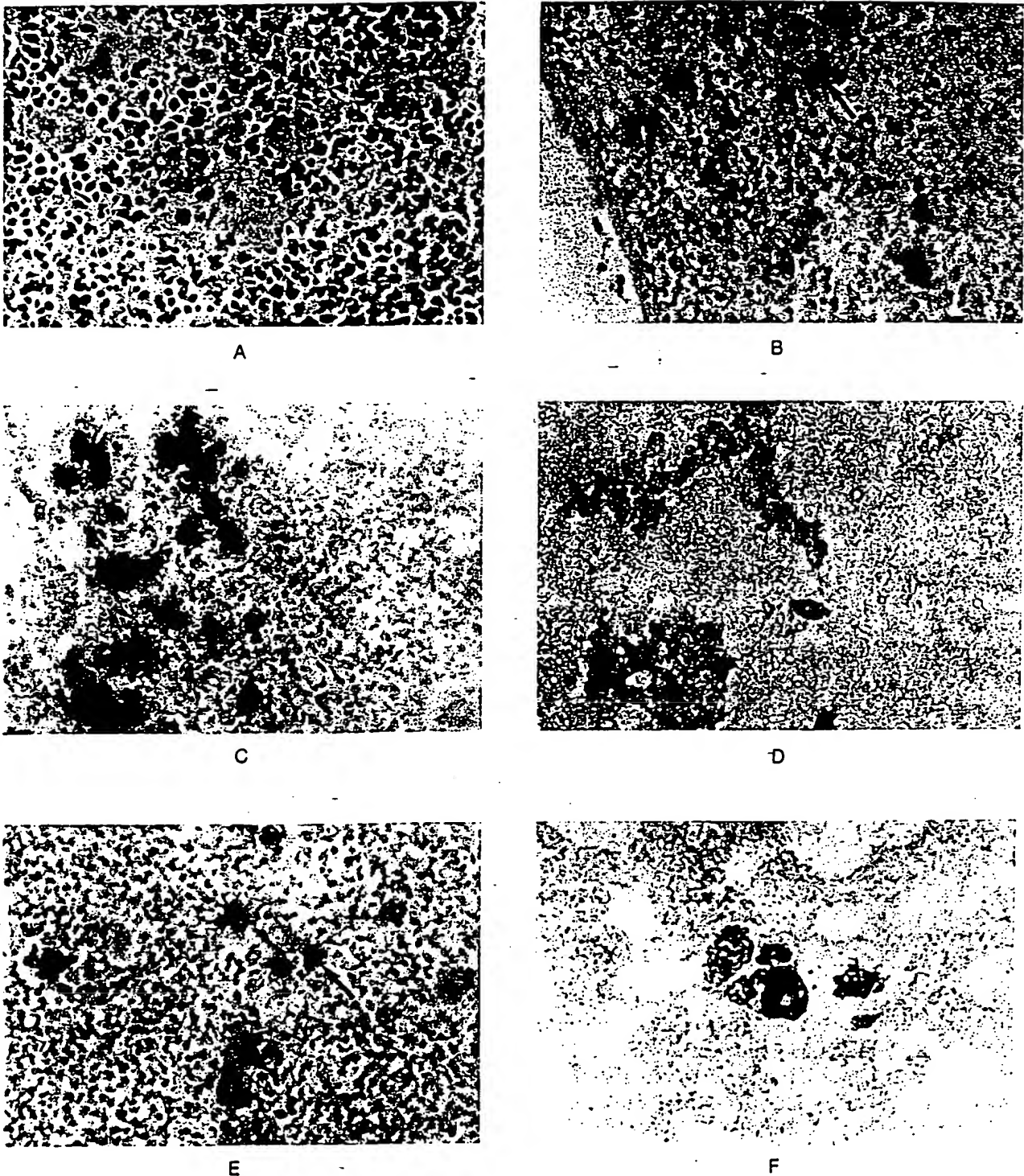


Fig. 2. Engraftment and differentiation of DPPIV⁺ hepatoblasts in the spleen of DPPIV⁻ recipients. (A) Hepatoblasts in spleen 5 d after transplantation. Hepatoblasts are located in the red pulp of the spleen and exhibit a morphology distinct from splenocytes (hematoxylin & eosin stain). (B) Hepatoblasts in the spleen of a recipient 5 d after transplantation. DPPIV is expressed on the surface of transplanted hepatoblasts (nuclei were counterstained with methyl green). One of the transplanted hepatocytes is shown with an arrow. (C) Transplanted hepatocytes after 8 weeks in the spleen of a recipient. Cells showed a similar morphology throughout the 8 week duration of the experiments. (D) G-6-P expression in transplanted hepatoblasts in spleen. (E) Morphology of adult DPPIV⁺ hepatocytes 1 week after transplantation into the spleen of a DPPIV⁻ recipient. Note that transplanted hepatocytes appear similar to transplanted hepatoblasts (methyl green counterstain). One of the transplanted hepatocytes is marked by an arrow. (F) G-6-P expression in adult rat hepatocytes transplanted into spleen.

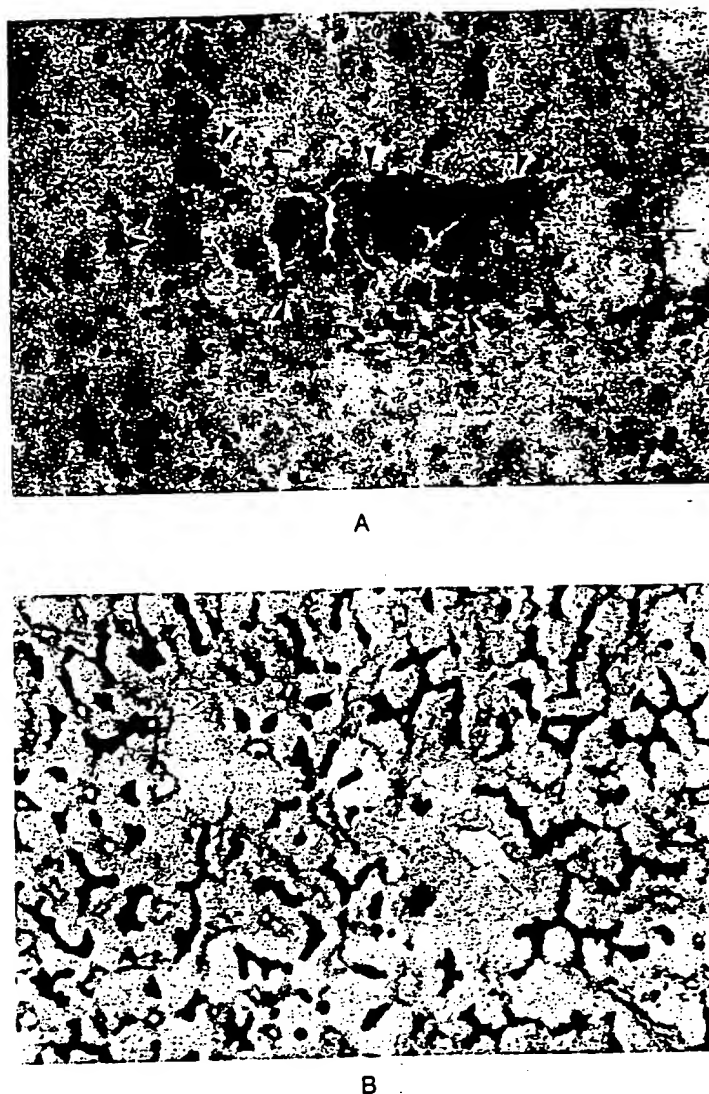


Fig. 3. Transplanted DPPIV⁺ hepatoblasts in the liver of a DPPIV⁻ recipient. (A) The liver of a DPPIV⁻ recipient 8 weeks after cells were injected into the spleen. A cluster of DPPIV⁺ transplanted hepatoblasts is enclosed by arrowheads. Some hepatoblasts remained within hepatic sinusoids, whereas others entered host liver plates. (B) Normal donor liver from a Fischer 344 adult rat demonstrating DPPIV activity in bile canaliculi.

cellular differentiation. This is especially important as early progenitor cells may differentiate into multiple phenotypes as seen with bipotent hepatoblasts (Germain et al., 1988a; Shiojiri, 1984). Using early embryonic mouse liver fragments, Shiojiri (1984) showed that the local microenvironment influenced the final phenotype: when implanted in testis, hepatoblasts preferentially differentiated into hepatocytes and when deposited subcutaneously, into bile ducts. Although precise mechanisms of cell differentiation in embryonic liver fragments or our studies were unclear, interactions with other cell types, local growth factors or differentiating agents, and extracellular matrix components are likely. In vitro studies demonstrate that promoters of differentiated function in adult hepatocytes,

e.g., cortisol, dimethyl sulfoxide, sodium butyrate, and extracellular matrix components, also induce differentiation in progenitor cells (Germain et al., 1988a; Sirica et al., 1990). Thus, organs that promote differentiation in vivo of precursor cells should be the same as those that preserve the differentiated state of adult hepatocytes.

Insight toward these ends may be derived from hepatocyte transplantation studies. Primary hepatocytes have been transplanted into myriad sites, including liver, spleen, dorsal fat pad, peritoneum, pancreas, renal capsule, and lung (reviewed in Gupta and Roy Chowdhury, 1994). Besides liver, the spleen among various ectopic sites best supports permanent hepatocyte engraftment and expression of hepatocyte specific

genes (Gupta et al., 1994; Maganto et al., 1990). The dorsal fat pad, in contrast, is less suitable as hepatocytes at this site exhibit rapid dysregulation of gene expression. However, biliary cells survive and readily form well-differentiated ductular structures in the dorsal fat pad (Sirica et al., 1985).

Continued differentiation of hepatoblasts already committed to the hepatocyte lineage in spleen indicates the presence of appropriate conditions in this organ, as found also in the testis (Lamers et al., 1990; Shiojiri et al., 1991). On the other hand, early, bipotent hepatoblasts differentiate along the biliary lineage in the dorsal fat pad. Similarly, preneoplastic hepatocytes transplanted into liver or spleen preserve well differentiated cellular morphology (Goyette et al., 1990; Hunt et al., 1985; Lee et al., 1981, 1982, 1983). In contrast, the subcutaneous tissue or dorsal fat pad yield less favorable results as transplanted preneoplastic cells survive poorly and the tumors that do arise are not well differentiated (Ohmori et al., 1980; Williams et al., 1977, 1980). The fate of activated, nonparenchymal epithelial liver cells, including oval cells, also varies in a site-specific manner. When transplanted into subcutaneous tissue or the dorsal fat pad, anaplastic carcinomas or adenocarcinomas, consistent with a biliary lineage, develop most commonly (Braun et al., 1987; Coleman et al., 1993; Fausto, 1990; Garfield et al., 1988; Goyette et al., 1990; Tsao and Grisham, 1987a; Williams et al., 1973). In contrast, nonparenchymal epithelial liver cells that form spindle cell tumors in subcutaneous tissue differentiate into hepatocytes in liver (Coleman et al., 1993; Tsao and Grisham, 1987b).

The factors which permit hepatocyte differentiation in the liver and spleen remain to be determined and were not addressed in our studies. Among possible explanations, extracellular matrix (ECM) components may play a critical role. Maintenance of the normal phenotype in adult hepatocytes requires adhesion of cells to an appropriate ECM (Rana et al., 1994). Differentiation of hepatoblasts along a specific lineage (biliary) may also involve interactions with specific ECM components (Shah and Gerber, 1990). Since a sinusoidal morphology of endothelial cells in culture is dependent on the appropriate ECM (McGuire et al., 1992), the matrix in spleen, which also contains sinusoids, might substitute for liver matrix and play a role in differentiation of uncommitted hepatic progenitor cells, but this requires further study.

A bioassay must allow localization and unequivocal identification of the transplanted cells. Following intrahepatic transplantation, donor progenitor cells have been recognized by persistence of GGT activity, a semiallogeneic transplantation protocol utilizing the RT1 Class I major histocompatibility complex, and

labeling with the *Escherichia coli* lac Z reporter gene or a fluorescent membrane dye (Coleman et al., 1993; Hanigan and Pitot, 1985; Hunt et al., 1982, 1985; Laishes and Farber, 1978). In contrast with our approach, however, these methods to localize cells have limitations, including possible cell modification, decreased cell viability, artifacts with contaminating helper viruses, or confusion with background staining (Hixson and Faris, 1994). As noted, the ectopic splenic location permitted identification of G-6-P positive cells as donor, a status confirmed by their coexpression of DPPIV as reported for adult hepatocytes (Gupta et al., 1995; Thompson et al., 1991). As DPPIV⁻ animals totally lacked DPPIV activity, transplanted cells could be unequivocally localized. The expression of DPPIV activity may further serve as a maturational marker in transplanted DPPIV⁺ cells of the hepatocyte phenotype (Feracci et al., 1987; Hong et al., 1989). Moreover, the use of weanling recipients allowed identification of small numbers of transplanted cells, and transplantation of syngeneic cells avoided allograft rejection. The importance of the hepatic microenvironment in differentiation of liver stem cells was recently emphasized (Coleman et al., 1993; Grisham et al., 1993). We demonstrate here that the spleen is also conducive to hepatocyte differentiation. The application of our or similar bioassays to transformed or nontransformed liver cells should facilitate analysis of hepatocyte differentiation and hepatic oncogenesis.

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